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(54) **Title:** TISSUE INHIBITOR OF MATRIX METALLOPROTEINASES TYPE-1 (TIMP-1) AS A POSTOPERATIVE MARKER FOR MINIMAL RESIDUAL DISEASE OR RECURRENT DISEASE IN PATIENTS WITH A PRIOR HISTORY OF CANCER

(57) **Abstract:** The present invention describes a method for determining whether an individual is suffering from minimal residual diseases or recurrent cancer during treatment and/or after being treated for the primary cancer by determining a parameter representing the post-operative TIMP-1 concentration in body fluid samples from the individual. In addition, the invention describes the additive effect of combined post-operative measurements of plasma TIMP-1 and serum CEA.

TISSUE INHIBITOR OF MATRIX METALLOPROTEINASES TYPE-1 (TIMP-1) AS A POSTOPERATIVE MARKER FOR MINIMAL RESIDUAL DISEASE OR RECURRENT DISEASE IN PATIENTS WITH A PRIOR HISTORY OF CANCER

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a test to be used to monitor colorectal cancer patients for the persistency of minimal residual disease (MRD) or for the recurrence of their cancer disease. The method is based on the measurement of tissue inhibitor of metalloproteinases 1 (TIMP-1) in body fluids either alone or in combination with other tumor markers, e.g. Carcino Embryonic Antigen (CEA). The invention permits the early identification of which patients have persistent MRD or which patients who will experience recurrence of colorectal cancer, either as local recurrence or as distant metastases.

The test is based on the measurement of tissue inhibitor of metalloproteinases type 1 (TIMP-1), in various body fluids, including blood, plasma, serum, saliva, stool, cerebrospinal fluid and urine. TIMP-1 concentrations can be determined either as the total TIMP-1 concentration, the free TIMP-1 concentration, the concentration of complexes between TIMP-1 and Matrix Metallo-Proteinases (MMPs) and/or ratios and fractions thereof, hereafter referred to as TIMP-1 levels. According to the invention, individuals with a prior diagnosis of cancer who have a high likelihood of later developing clinically manifest recurrent cancer, e.g. gastrointestinal cancer such as colon cancer, can be identified by elevated postoperative TIMP-1 levels in their body fluids, while individuals with low postoperative TIMP-1 levels are unlikely to suffer from or later develop recurrent cancer. Thus, the invention can be used to identify individuals with a high probability of having or later developing recurrent colorectal cancer. The identified individuals should be further examined and if recurrent cancer or minimal residual disease (MRD) or both recurrent cancer and MRD are found, the patients should be offered surgery, irradiation, anti-neoplastic therapy or any combination thereof, thereby increasing the chance of cure and/or long term survival of the individual.

BACKGROUND

Colorectal cancer is the fourth most frequent cancer in the Western world, with about 160,000 new cases yearly in the US. Forty to 50% of all colorectal cancer patients will be diagnosed with early stage disease (Dukes´ stage A or B). Most of these patients with early stage colorectal cancer can be cured by surgery alone. Thus, risk of recurrence is closely related to stage of disease at time of primary surgery, with about a 10% relapse

rate in Dukes' stage A and 25-30% in Dukes' stage B. Patients with Dukes' stage C colorectal cancer have a five-year relapse rate of 70% following surgery and are offered adjuvant chemotherapy.

Thus, 30-40 percent of patients who have undergone a complete resection of a colorectal malignancy will experience relapse with metastatic disease, which is usually fatal. Thus, hundreds of thousands of people with resected CRC (colorectal cancer) are candidates for surveillance, as it is a commonly held clinical view that early detection of metastases and metachronous disease by relevant surveillance regimens may allow for interventions with the aim of improving overall survival, disease-free survival and quality of life.

The majority of recurrences of CRC occur within 5 years, and usually within 3 years following surgery. A tremendous effort has been put into the establishment of effective tests for the detection of recurrent disease at a stage when intervention is not futile. Carcinoembryonic antigen (CEA) tests, colonoscopies, chest x-rays, liver function tests, complete blood cell counts, fecal occult blood tests, computerized tomography (CT), ultrasonography, magnetic resonance (MR) and positron emission tomography (PET) are all methods that have been extensively reported on for the postoperative surveillance of CRC. Unfortunately, the results from randomised studies of such monitoring of CRC patients have varied widely and demonstrated minimal efficacy (Schoemaker, Gastroent, 1998; Ohlsson, Dis Col Rec, 1995; Makela, Arch Surg, 1996; Kjeldsen, Int J Color Dis, 1997). As a result hereof, considerable variation in clinical follow-up practice is evident (Virgo KS, Ann Surg, 1995) and expenses for 5-year follow-up of CRC patients have differed from $561 to $ 16,492 per patient (Virgo, JAMA, 1995).

The American Society of Clinical Oncology (ASCO) has recommended against the use of liver function tests, fecal occult blood tests, complete blood counts, pelvic imaging, CT scanning and chest x-rays as means for regular postoperative monitoring of CRC. However, it was recommended that postoperative measurement of serum CEA is performed every 2 to 3 months for ≥ 2 years in patients with stage II or III disease where resection of liver metastases is clinically indicated (Benson, JCO, 2000). Of note, however, is the fact that approximately 30 percent of all CRC recurrences do not produce CEA (Safi, Cancer Detect Prev, 1993). Thus, it was stressed by the ASCO that new surveillance methods for the detection of CRC recurrences are needed (Benson, JCO, 2000). This statement has gained further weight as recent data has shown that chemotherapy of metastatic CRC can improve short-term survival and often improve the quality of life (Cunningham, Lancet, 1998) and that treatment of asymptomatic CRC yields better results than postponing treatment until the disease has become symptomatic (Nordic Gastr Tumor Adj Group, JCO, 1992).

Because metastatic disease is the main cause of cancer patient morbidity and mortality, molecules involved in the regulation of tumor invasion and metastasis are attractive as potential diagnostic/prognostic/monitoring targets. It is well established that proteolytic enzymes produced by cancer cells or by cells in the tumor stroma are involved in extracellular tissue degradation, leading to cancer cell invasion and metastasis. A number of enzymes have been associated with this process, the most thoroughly investigated being the metalloproteinases, such as the collagenases and stromelysins, and the serine proteases such as plasmin. Recently, data have been published indicating that these molecules, free or bound in complexes, are released from tumor tissue and find their way into the circulation.

Matrix metalloproteinases (MMP's) play a pivotal role in cancer growth and spread, contributing to enzymatic degradation of the extracellular matrix (Liotta *et al*, 1991; Stetler-Stevenson *et al*, 1993; MacDougall & Matrisian, 1995). The naturally occurring inhibitors of MMP's, tissue inhibitors of MMP's (TIMP's), form tight 1:1 stoichiometric complexes with the activated forms of the MMP's (Welgus *et al*, 1985; Kleiner *et al*, 1993), thereby inhibiting the catalytic activity of these enzymes (Stetler-Stevenson *et al*, 1996; Goldberg *et al*, 1992; Birkedal-Hansen *et al*, 1993). While the balance between the matrix-degrading properties of MMP's and the inhibitory effect of TIMP's is closely regulated under normal physiological conditions (Matrisian, 1992; Thorgeirsson *et al*, 1993; Birkedal-Hansen *et al*, 1993), this balance might be disrupted in malignant tissue.

A number of enzyme-linked immunoassays for the detection of TIMP-1 (Kodama *et al*, 1989; Cooksley *et al*, 1990; Clark *et al*, 1991) and TIMP-2 (Fujimoto *et al*, 1993) have been described. These assays have been applied to body fluids, e.g. serum, plasma, amniotic fluid, cerebrospinal fluid, urine, but the number of samples tested has not been sufficient to establish normal ranges for TIMP levels in healthy individuals (Kodama *et al*, 1989; Clark *et al*, 1991). Furthermore, none of these assays have been sufficiently validated for technical performance or for clinical use. We have recently described and validated an ELISA for the quantitation of total TIMP-1 in plasma samples, and using this assay we could demonstrate that healthy blood donors have a very narrow range of total plasma TIMP-1 (Holten-Andersen et al, Br. J.Cancer 1999). We have also recently developed and validated an ELISA for the quantitation of uncomplexed TIMP-1 in plasma (Holten-Andersen et al., Clin. Chem 2002)

In a study by Mimori et al (Mimori *et al*, 1997) in which tumor tissue levels of TIMP-1 mRNA were studied in patients with gastric carcinoma, high tumor/normal tissue ratios of

TIMP-1 mRNA were found to be associated with increased invasion and poor prognosis and Guillem *et al.* (Proc Annu Meet Am Assoc Cancer Res; 34:A466, 1993) suggest a possible correlation between TIMP-1 RNA levels and poorly differentiated, invasive colorectal cancers. However, TIMP-1 protein levels in sera from prostate cancer patients and healthy donors (Baker *et al*, 1994) showed a high degree of overlap. Similarly, a separate study of plasma from prostate cancer patients and healthy donors showed no difference in TIMP-1 levels between the two groups (Jung *et al*, 1997).

We have recently published that preoperatively measured plasma levels of total TIMP-1 show highly significant association with colorectal cancer patient survival, i.e. patients with high TIMP-1 levels had a significantly shorter survival than those patients with low TIMP-1 levels (Holten-Andersen et al., Clin. Cancer Res, 2000 and WO 00/62070).

Furthermore, Nauro *et al.* (J. Urol, vol. 1, no 3, September 1994, pp 228-231) found that serum TIMP-1 levels are significantly higher in metastatic bladder cancer patients compared to a healthy control group, whereas no results were presented about the TIMP-1 levels of pre-metastatic bladder cancers. TIMP-1 measurements in urine have also been described as being useful in the diagnosis of other cancers such as bladder cancer, liver cancer and renal cancer (Japanese patent application no. 8-136548).

Measurements of TIMP-1 levels have also been shown to be useful in diagnosing diseases of the nervous system as described in US 5,324,634.

Studies of TIMP-1 complexed with MMP-9 in plasma of patients with advanced gastrointestinal and gynaecological cancer (Zucker *et al*, 1995) demonstrated significantly higher levels in blood samples from cancer patients with metastatic disease compared to healthy control individuals, and that patients with high levels of TIMP-1:MMP-9 complex had a shorter survival (Zucker *et al*, 1995 and US 5,324,634). However, this study did not include measurements of total or free TIMP-1, only the complex between TIMP-1 and one of the up to now approximately 24 identified MMP's. Furthermore, in this study, no differences in complex levels were found between patients with breast cancer and healthy donors. Also, this study did not include patients with early stage cancer.

DETAILED DESCRIPTION OF THE INVENTION

In a number of cancer types, there is a critical and unmet need for highly sensitive and specific markers for early detection of MRD or recurrent cancer disease. Such markers are able to identify individuals with a high risk of recurrent cancer. If positive test results are obtained, these individuals should be further examined, and if minimal residual disease

(MRD) or recurrent cancer or both MRD and recurrent cancer is found, the patients should subsequently be offered surgery, radiation, systemic anti-neoplastic therapy or any combination thereof, e.g. both surgery and radiation, surgery and systemic anti-neoplastic therapy (chemotherapy).

Since proteinases and their receptors and inhibitors seem to play a pivotal role in the basic mechanisms leading to cancer invasion, these molecules may be expressed at a very early time point in the invasion and metastasis process. As many of these molecules exert their biological action extracellularly, they may be present at elevated levels in body fluids, even in patients with minimal residual disease after the primary treatment.

The present invention relates to a method to aid in the diagnosis of MRD or recurrent cancer or both MRD and recurrent cancer in a patient, said method comprising determining the amount of total, complexed and/or free TIMP-1 levels and ratios and fractions thereof in body fluids such as blood, serum, plasma, saliva, urine, faeces or cerebrospinal fluid. These measurements can be combined with measurements of CEA or other markers of recurrent colorectal cancer and additive diagnostic effect may thus be obtained.

Gastrointestinal cancers such as colon cancer, rectal cancer, colorectal cancer, pancreatic cancer, stomach (Gastric) cancer, esophageal cancer, liver cancer or bladder cancer are preferred types of cancer wherein the present method can be applied.

An aspect of the present invention relates to a method for determining whether an individual is likely to have MRD or recurrent gastrointestinal cancer, such as colorectal cancer, the method comprising determining a first parameter representing the concentration of TIMP-1 in body fluid samples, and indicating the individual as having a high likelihood of having MRD or recurrent cancer or both MRD and recurrent cancer if the parameter is at or beyond a discriminating value and indicating the individual as unlikely of having recurrent cancer or MRD if the parameter is not at or beyond the discriminating value.

The parameter representing the concentration of TIMP-1 may be the concentration proper of TIMP-1. TIMP-1 exists both in free form and in the form of complexes with MMPs, and it has been found that an important parameter is the total concentration of TIMP-1, that is, the sum of the TIMP-1 in free form and the TIMP-1 in complex forms. It will be understood that the other expressions than the concentration proper can represent the concentration, such as, e.g., the concentration multiplied by a factor, or similar expressions, and that such other representations can be used equally well for the purpose of the present invention provided the corresponding adjustments are made.

The discriminating value is a value which has been determined by measuring the parameter in both a healthy control population and a population with known MRD or recurrent cancer thereby determining the discriminating value which identifies the cancer population with either a predetermined specificity or a predetermined sensitivity based on an analysis of the relation between the parameter values and the known clinical data of the healthy control population and the cancer patient population, such as it is apparent from the detailed discussion in the examples herein. The discriminating value determined in this manner is valid for the same experimental setup in future individual tests.

Preferred embodiments of the present invention are described below.

The present invention relates in general to a method for determining whether an individual is likely to have minimal residual disease and/or recurrent cancer after being treated for the primary cancer, said primary cancer being the possible progenitor to the minimal residual disease or recurrent cancer, the method comprising determining a first parameter representing the post-operative concentration of TIMP-1 in body fluid samples, and indicating the individual as having a high likelihood of having minimal residual disease and/or recurrent cancer if the parameter is at or beyond a discriminating value and indicating the individual as unlikely of having minimal residual disease or recurrent cancer if the parameter is not at or beyond the discriminating value.

In a preferred embodiment the discriminating value is a value which has been determined by measuring said at least one first parameter in both a healthy control population and in a population with known minimal residual disease and/or recurrent cancer, thereby determining the discriminating value which identifies the minimal residual disease and/or recurrent cancer population with a predetermined specificity or a predetermined sensitivity.

In an equally preferred embodiment the discriminating value is a value which has been determined by measuring said at least one first parameter in a healthy control population, thereby determining the discriminating value which identifies individuals with increased plasma TIMP-1 values e.g. above or equal to the 95th percentile of healthy control individuals. The 95th percentile is meant to be the TIMP-1 value which dichotomises the individuals so that 5% have higher TIMP-1 levels and 95% lower TIMP-1 levels. Thus, the individuals with increased plasma TIMP-1 values are those having TIMP-1 values equal to or greater than 95% of the healthy control individuals.

In an additional embodiment the present invention relates to a method for determining whether an individual is likely to have minimal residual disease and/or recurrent cancer after being treated for the primary cancer, said primary cancer being the possible pro-

genitor to the minimal residual disease or recurrent cancer, the method comprising determining the post-operative level of a first parameter representing the concentration of TIMP-1 in one or more body fluid samples, and indicating the individual as unlikely of having minimal residual disease and/or recurrent cancer if the first parameter is below a discriminating value where this discriminating value is the pre-operative level of said first parameter representing the concentration of TIMP-1 in one or more body fluid samples and indicating the individual as having a high likelihood of having minimal residual disease or recurrent cancer if the first parameter is not below this discriminating value.

The phrase "below the discriminating value" is meant to identify the situation where the post-operative level of said first parameter is at least 0.001% below the pre-operative level, such as at least 0.01% below, e.g. at least 0.1% below, e.g. at least 1% below the pre-operative level.

Furthermore the post-operative level of said first parameter may be obtained at any time after the operation, such as at least 1 day post-operatively, such as 5 days post-operatively, e.g. 1 week post-operatively, e.g. 2 weeks post-operatively, e.g. 1 month post-operatively, e.g. 1.5 month post-operatively, e.g. 2 months post-operatively, e.g. 3 months post-operatively, e.g. 4 months post-operatively, e.g. 5 months post-operatively, e.g. 6 months post-operatively, e.g. 7 months post-operatively, such as 8 months post-operatively, such as 12 months post-operatively.

In an additional embodiment the present invention relates to a method for following a patient after the primary operation by longitudinal measurements. These measurement may be of either TIMP-1 (total TIMP-1, free TIMP-1 or complexed TIMP-1) alone or in combination with at least one second parameter whereby the detection of recurrent disease can be facilitated.

It is obvious that the determination of TIMP-1 level with advantage can be performed at several time points at intervals as part of the monitoring of a cancer patient after the treatment for primary cancer for as long as it is found relevant, and that this recurrent determination of the TIMP-1 level can be performed according to any one of the herein described embodiments.

In a preferred embodiment the first parameter determined is the total concentration of TIMP-1.

In a further preferred embodiment the at least one first parameter determined is the value obtained by combining the concentration of total TIMP-1 with the concentration of free

TIMP-1, alternatively the at least one first parameter determined may be the value obtained by combining the concentration of total TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP or the at least one first parameter determined may be the value obtained by combining the concentration of free TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP. The combined values are obtained by performing logistic regression analysis.

A special embodiment of the present invention is the method which comprises additionally determining at least one second parameter, the second parameter representing the concentration of an additional marker different from any form of TIMP-1, in a body fluid sample from the individual.

This results in a method wherein the first parameter representing the concentration of TIMP-1 in body fluid samples and the at least one second parameter different from any form of TIMP-1 are combined to result in a combined parameter and indicating the individual as having a high likelihood of having minimal residual disease and/or recurrent cancer if the combined parameter is at or beyond a discriminating value and indicating the individual as unlikely of having minimal residual disease and/or recurrent cancer if the combined parameter is not at or beyond the discriminating value.

Again, the combined values are obtained by performing logistic regression analysis.

In the embodiment where at least one second parameter is combined with measurement of TIMP-1, the discriminating value of the combined parameter may be a value which has been determined by determining said combined parameter in both a healthy control population and a population with known minimal residual disease and/or recurrent cancer, thereby determining the discriminating value which identifies the population with minimal residual disease and/or recurrent cancer with a predetermined specificity or a predetermined sensitivity

Furthermore, the discriminating value of the combined parameter may be a value which has been determined by measuring said combined parameter in a healthy control population, thereby determining the discriminating value which identifies individuals with increased values of the combined parameter e.g. above or equal to the 95th percentile of healthy control individuals. Thus, the individuals with increased combined parameter values are those having combined parameter values equal to or greater than 95% of the healthy control individuals.

A further embodiment of the present invention is a method wherein the first parameter representing the concentration of TIMP-1 in body fluid samples and the at least one second parameter different from any form of TIMP-1 are combined to result in a combined parameter and indicating the individual as unlikely of having minimal residual disease and/or recurrent cancer if the post-operative level of the combined parameter is below the pre-operative level of said combined parameter and indicating the individual as having a high likelihood of having minimal residual disease and/or recurrent cancer if the post-operative level of the combined parameter is not below the pre-operative level of said combined parameter.

The phrase "below the discriminating value" is meant to identify the situation where the post-operative level of the combined parameter is at least 0.001% below the pre-operative level, such as at least 0.01% below, e.g. at least 0.1% below, e.g. at least 1% below the pre-operative level

Again, the post-operative level of said combined parameter may be obtained at any point after the operation, such as at least 1 day post-operatively, such as 5 days post-operatively, e.g. 1 week post-operatively, e.g. 2 weeks post-operatively, e.g. 1 month post-operatively, e.g. 1.5 month post-operatively, e.g. 2 months post-operatively, e.g. 3 months post-operatively, e.g. 4 months post-operatively, e.g. 5 months post-operatively, e.g. 6 months post-operatively, e.g. 7 months post-operatively, such as 8 months post-operatively, such as 12 months post-operatively.

In a preferred embodiment of the present invention, the at least one second parameter determined is a parameter representing the concentration of a tumour marker where the tumour marker is selected from the group consisting of CEA, soluble u-PAR, cathepsin B, cathepsin H, HER2-neu, CA15-3 and YKL-40, 19.9 and CA242. In a more preferred embodiment, the at least one second parameter determined is the concentration of CEA.

In general, the method of the present invention relates to the situation where the individual may be a member of a population already identified as having an increased risk of developing recurrent cancer.

It is obvious that the determination of the TIMP-1 level or the determination of the combination of TIMP-1 level and the level of a non-TIMP-1 marker may be performed at several time points at intervals as part of a monitoring of a cancer patient after the treatment for primary cancer.

The determination of the concentration of the markers, either TIMP-1 or any of the non-TIMP-1 markers may performed by means of an immuno assay, such as ELISA, or an activity assay, such as zymography.

In an additional embodiment of the present invention the determination of the TIMP-1 level or the determination of the combination of TIMP-1 level and the level of a non-TIMP-1 marker may be used to follow a patient or a group of patients receiving adjuvant treatment and thereby detect treatment failure early and thus allowing an early change in treatment modality. By using the measurements of the present invention, patients who do not respond by a decrease in the levels of TIMP-1 and/or the level of non-TIMP-1 marker(s) upon a few number of cycles of (e.g. 2 or 3 cycles) of the adjuvant treatment can be considered as non-responders and should therefore be taken off treatment and offered an alternative systemic treatment.

Thus, one embodiment of the present invention comprises a method of monitoring cancer patients receiving post-operation adjuvant anti-neoplastic treatment for a cancer. In detail the method comprises determining at least one first parameter representing a concentration of TIMP-1 in body fluid samples after at least one cycle of adjuvant anti-neoplastic treatment which indicates the individual as not responding to the adjuvant anti-neoplastic treatment if this first parameter is at or beyond a discriminating value. The discriminative value is in this embodiment a post-operative concentration of TIMP-1 obtained before the first cycle of adjuvant anti-neoplastic treatment is initiated, typically 1-2 weeks after operation, such as between 1 and 30 days after operation. The method will indicate the individual as responding to the adjuvant anti-neoplastic treatment if the parameter is not at or beyond the post-operative concentration of TIMP-1 obtained before the first cycle of adjuvant systemic anti-neoplastic treatment.

The adjuvant anti-neoplastic treatment may be a adjuvant systemic anti-neoplastic treatment and the cancer may a primary cancer.

The least one first parameter representing the concentration of TIMP-1 is selected from the group consisting of the total concentration of TIMP-1, the value obtained by combining the concentration of total TIMP-1 with the concentration of free TIMP-1, the value obtained by combining the concentration of total TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP and the value obtained by combining the concentration of free TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP.
The method of following a patient or a group of patients receiving adjuvant treatment may also comprise additionally determining at least one second parameter, the second parameter representing the concentration of an additional marker different from any form

of TIMP-1 and selected from the group consisting of CEA, soluble u-PAR, any fractions of soluble u-PAR, cathepsin B, cathepsin H, HER2-neu, CA15-3 and YKL-40, 19.9 and CA242, in a body fluid sample from the individual. The at least one first parameter representing the concentration of TIMP-1 in body fluid samples and the at least one second parameter different from any form of TIMP-1 may be combined to result in a combined parameter which indicates the individual as not responding to the adjuvant anti-neoplastic treatment if this first parameter is at or beyond the discriminating value.

All of these combinations being performed by logistic regression analysis.

"Sensitivity" is defined as the proportion of positives (i.e. individuals having a parameter representing the concentration of a marker, such as TIMP-1 in body fluid samples higher than a predefined diagnostic level) that are correctly identified by the described method of the invention as having MRD and/or recurrent cancer. "Specificity" is defined as the proportion of negatives (i.e. individuals having a parameter representing the concentration of a marker, such as TIMP-1 in body fluid samples lower than a predefined diagnostic level) that are correctly identified by the described method as not having the disease.

The invention may be used both for an individual and for an entire population.

In the specific experimental setups described herein, the concentration threshold of total TIMP-1 useful as a discriminating value was found to be 112 microgram/L of total TIMP-1 which is the 95th percentile of healthy donors. Other experimental setups and other parameters will result in other values (specificities/sensitivities) which can be determined in accordance with the teachings herein.

An important point is that the present invention is not directed to such a single, precise value of TIMP-1 concentration representing the discriminating value. A relevant discriminating value of the TIMP-1 concentration can be selected from ROC (Receiver Operating Characteristics) curves which are disclosed e.g. in figure 14. These curves give the correlation between sensitivity and specificity and the sensitivity/specificity for any selected total TIMP-1 threshold value can be derived from the curves. A threshold value resulting in a high sensitivity results in a lower specificity and vice versa. For example, if one wishes to detect all colorectal cancers with a high degree of certainty, then the specificity will be lower and some false positives are likely to be included. If one wishes to be relatively certain that only malignant colorectal cancers will be detected, then a number of colorectal cancers are likely not to be identified.

Using figure 8 which represents plasma TIMP-1 values of 807 healthy blood donors (lower curve) and 588 colorectal patients (upper curve), one can take the 95 percentile of the donors (x-axis = 95%) and read the corresponding plasma TIMP-1 concentration for the healthy blood donors directly on the y-axis. By taking the 95 percentile of the donors, the specificity of plasma TIMP-1 for colorectal cancer in a population corresponding to the included blood donors will be 95% (e.g. there will be 5% of the donors having a plasma TIMP-1 concentration above this value). Using this plasma TIMP-1 value, the sensitivity of plasma TIMP-1 can be read by finding this value on the y-axis of figure 8 and then make a horizontal line. Where the horizontal line intersects with the percentile curve of the colorectal cancer patients (upper curve in figure 8), the sensitivity can be read by drawing a vertical line and read the sensitivity in percentage on the x-axis.

Each individual diagnostic department or a person thus can determine which level of sensitivity/specificity is desirable and how much loss in specificity is tolerable. The chosen discriminating value could be dependent on other diagnostic parameters used in combination with our claimed method by the individual diagnostic department, e.g. the use of coloscopy.

The method can be applied to all patients who have had primary treatment for colorectal cancer. When an individual has been identified as having high TIMP-1 levels in his or her body fluid, the individual should be referred for further examination. If MRD and/or a recurrent cancer is found, the patient could be offered surgery, radiation and/or systemic anti-neoplastic therapy or any combination thereof aiming at curing the patient of cancer.

Example 1 describes the preparation and validation of an assay that measures total TIMP-1 with high analytical sensitivity and specificity. It is described that healthy blood donors have a very narrow range of plasma total TIMP-1. This assay was used to measure plasma TIMP-1 on 807 blood donors.

In Example 2, the formatting of a TIMP-1:MMP-9 ELISA is described. The format, execution, and validation of this assay are similar to those for total TIMP-1, except that a polyclonal antibody against MMP-9 is used in the capture step. By substituting the MMP-9 antibody with an antibody against another MMP, complexes between TIMP-1 and other MMP's can be quantitated.

In Example 3, the formatting of a TIMP-1 assay which exclusively measures free TIMP-1, is described. This assay utilizes a monoclonal anti-TIMP-1 antibody (MAC 19) which only recognises TIMP-1 in its uncomplexed form. Thus, this assay will measure the amount of

free TIMP-1 in a sample. The execution and validation of this assay are similar to the assay for total TIMP-1

By subtracting the free TIMP-1 concentration from the total TIMP-1 concentration in a biological sample, the concentration of all complexed forms of TIMP-1 can be determined. It should be emphasized, however, that TIMP-1 can form complexes with many of the MMP's and therefore, subtracting one type of complex from the total amount of TIMP-1 will only provide information on the fraction of TIMP-1 not being complexed to this specific MMP.

In Example 4, it is shown that a proportion of patients who have previously had surgery for primary colorectal cancer may have elevated plasma TIMP-1 levels 6 month post-surgery.

Example 5 shows that as an alternative to a fixed plasma TIMP-1 threshold value, patients with a high likelihood of MRD or recurrent cancer can also be identified as those patients who do not show a significant decrease in plasma TIMP-1 levels between the pre-operative plasma sample and the post-operative plasma sample.

In Example 6, it is shown that the post-operative information on recurrence probability obtained by measuring plasma TIMP-1 6 months postoperatively, is independent of Dukes classification, since statistically significant information on survival can be obtained in each of Dukes stage A+ B and C disease.

In example 7, it is shown that statistically significant information on metastasis-free survival and overall survival can be obtained by measuring plasma TIMP-1 and serum CEA 6 months post-operatively. However, the information obtained by simultaneous measuring plasma TIMP-1 and serum CEA is additive, e.g. significant additive information is gained when combining these to serological markers.

The clinical value of a marker for monitoring cancer patients is related to its ability to detect recurrence of the disease at an early time point when the patients still have no symptoms or clinical signs of disease recurrence. From the metastasis-free survival curves and the overall survival curves presented, it can be deduced that plasma TIMP-1 measurements yields highly statistically significant information on recurrence probability several months before clinically evident recurrence is observed. Thus, using TIMP-1, either alone or in combination with CEA, will result in more patients being identified at an early time point as having high probability of disease recurrence and these patients might be offered systemic anti-cancer therapy. The benefit to the patient is that treatment can be introduced at an early time point where the tumor burden is still limited.

The specificity of a given cancer monitoring test is based on the efficiency of the test to identify only those patients suffering from recurrent cancer while patients suffering from non-malignant diseases should not be identified as false positive subjects. In the case of colorectal cancer, it is important that the test in question can also distinguish between malignant and non-malignant diseases of the colon and rectal. This is particularly important for diseases like Crohn's disease and ulcerative colitis, since patients with these diseases are at higher risk of developing cancer.

In Example 8 it is shown that total TIMP-1 levels are significantly higher in patients with recurrent colorectal cancer than in patients with inflammatory bowel diseases (IBD), showing that total TIMP-1 can be used to monitor for colorectal cancer recurrence in a population of patients with IBD. That TIMP-1 is not increased in non-malignant diseases is supported by a recent paper, (Keyser *et al*, 1999), demonstrating that patients with rheumatoid arthritis do not have increased plasma TIMP-1 levels. Also, by comparing total TIMP-1 levels among patients with IBD (excluding patients with clinically assessed acute active disease, n=4) and healthy blood donors, no significant differences in total plasma TIMP-1 levels were found (p=0.56), showing that these non-malignant diseases do not give false positive test results. In a recent paper by Holten-Andersen et al., 2002, it was shown that patients (n=322) with primary breast cancer do not present with elevated plasma-TIMP-1 levels.

TIMP-1 is known to exist either as the free molecule or in complex with MMP's, preferentially MMP-9. Measuring total TIMP-1, complexed TIMP-1 and free TIMP-1 will make it possible to validate each of these species for their potential monitoring value. In addition, it will be possible to calculate ratios or any derived algorithm between the different species which might provide additional monitoring value.

FIGURE LEGENDS

Figure 1: Kinetic assay for TIMP-1. Progress curves for the change in absorbance at 405 nm produced by hydrolysis of p-nitrophenyl phosphate by solid-phase bound alkaline phosphatase immunoconjugate. The data shown are generated by 4 individual assay wells treated with 4 different concentrations of purified recombinant TIMP-1; 10 µg/L (▽-▽), 2.5 µg/L (Δ-Δ), 0.63 µg/L (□ - □) and 0.16 µg/L (O-O). The lines shown have been fitted by simple linear regression.

Figure 2: TIMP-1 standard curve. Absorbance units for triplicate TIMP-1 standards in the range of 0 to 5 µg/L are collected automatically over 60 minutes, with readings taken at 405 nm every 10 min. Progress curves are computed for each well and the rates obtained

are fitted to a standard curve using a four-parameter equation of the form $y = d + [(a-d)/(1+(x/c)^b)]$. In the example shown, the four derived parameters had the following values: a=1.87, b=1.11, c=3.35, d=73.5. The correlation coefficient for the fitted curve is >0.999.

Figure 3: Recovery of signal from standard TIMP-1 added in increasing concentration to assay dilution buffer (□ - □), a 1:100 dilution of EDTA plasma pool (Δ-Δ), a 1:100 dilution of citrate plasma pool (∇-∇) and a 1:100 dilution of heparin plasma pool (O-O). The values shown are the means of triplicates. The correlation coefficient for each fitted curve is greater than 0.99.

Figure 4: Western blotting of immunoabsorbed patient plasma sample. Lane 1: standard TIMP-1; lane 2: eluate of patient citrate plasma sample diluted 1:10 and immunoabsorbed with sheep polyclonal anti-TIMP-1. Bands of non-reduced standard TIMP-1 and TIMP-1 isolated from plasma sample both appear just below 30 kDa.

Figure 5a: Percentiles plot for the level of TIMP-1 (µg/L) measured in citrate plasma (O) and EDTA plasma (Δ) from the same individual in a set of 100 volunteer blood donors.

Figure 5b: Linear regression plot for the level of TIMP-1 in citrate plasma samples compared with EDTA plasma samples from the same 100 individuals. The equation of the fitted line is y = 0.93x, with a regression coefficient of 0.99.

Figure 6: Percentiles plot for the levels of TIMP-1 (µg/L) measured in two sets of citrate plasma samples obtained by the same procedure from volunteer blood donors at different times. 100 samples from May =97 (Δ) and 94 samples from Sept =96 (□).

Figure 7: Percentile plot for the levels of TIMP-1 (µg/L) measured in 194 citrate plasma samples from volunteer blood donors and stratified by sex into 107 males (Δ) and 87 females (O).

Figure 8: 807 healthy

Figure 9: Graphical illustration of the TIMP-1:MMP-9 complex ELISA.

Figure 10: Graphical illustration of the free TIMP-1 ELISA.

Figure 11: A plate was coated with the polyclonal anti-MMP-9 antibody. Different concentrations of TIMP-1:MMP-9 complex, free MMP-9, free TIMP-1 and a blank control were

added. Only TIMP-1:MMP-9 complexes and free MMP-9 were bound by the capture polyclonal anti-MMP-9 antibody. MAC19 was then added for antigen detection. Neither TIMP-1:MMP-9 complex nor free MMP-9 were detected by MAC19, defining the specificity of this antibody for free TIMP-1.

Figure 12: A plate was coated with the polyclonal anti-MMP-9 antibody. Different concentrations of TIMP-1:MMP-9 complex, free MMP-9, free TIMP-1 and a blank control were added. Only TIMP-1:MMP-9 complexes and free MMP-9 were bound by the capture polyclonal anti-MMP-9 antibody. MAC15 was then added for antigen detection. Only TIMP-1:MMP-9 complex bound by the capture polyclonal anti-MMP-9 antibody was detected by MAC15. Free MMP-9 was not detected by MAC15.

Figure 13: Recovery of signal in the free TIMP-1 assay from standard TIMP-1 added in increasing concentration to assay dilution buffer (□ - □), a dilution of EDTA plasma pool (Δ-Δ), a dilution of citrate plasma pool (∇-∇), and a dilution of heparin plasma pool (O-O). The values shown are the means of triplicates. The correlation coefficient for each fitted curve is greater than 0.99.

Figure 14: Percentile plot of total TIMP-1 in colorectal cancer patients as measured 6 months postoperatively and in healthy control subjects.

Figure 15: Univariate survival analysis for total TIMP-1 in colorectal cancer patients. Preoperative and postoperative plasma TIMP-1 levels were scored as low or high based on the 95th percentile of plasma TIMP-1 in an age matched healthy control group. The individual survival curves are marked "0 0", "1 0", "0 1" and "1 1" respectively to identify 1) low level of pre-operative TIMP-1, low level of post-operative TIMP-1 ("0 0"); 2) high level of pre-operative TIMP-1, low level of post-operative TIMP-1 ("1 0"); 3) low level of pre-operative TIMP-1, high level of post-operative TIMP-1 ("0 1"); and 4) high level of pre-operative TIMP-1, high level of post-operative TIMP-1 ("1 1").

Figure 16: Univariate survival curves. The patients were dichotomised using the 95th percentile of plasma TIMP-1 levels in healthy blood donors. Patient samples: 6 months postoperative plasma TIMP-1 level. The Figure shows the survival curves for Dukes A+B patients and for Dukes C patients, respectively. The individual survival curves are marked "A+B 0", "A+B 1", "C 0" and "C 1" respectively to identify 1) Dukes A+B with low postoperative TIMP-1 level ("A+B 0"); Dukes A+B with high postoperative TIMP-1 level ("A+B 1"); Dukes C with low postoperative TIMP-1 level ("C 0"); and Dukes C with high postoperative TIMP-1 level ("C 1").

Figure 17: Univariate metastasis-free survival curves. The patients were dichotomised into four groups according to their 6 months postoperative plasma TIMP-1 level and serum CEA level. Group 1 ("0 0"): low TIMP-1 and low CEA; Group 2 ("1 0"): high TIMP-1 and low CEA; Group 3 ("0 1"): low TIMP-1 and high CEA; Group 4 ("1 1"): high TIMP-1 and high CEA. The patients were dichotomised using the 95th percentile of plasma TIMP-1 levels in healthy blood donors and 5 ng/ml of CEA.

Figure 18: Univariate overall survival curves. The patients were dichotomised into four groups according to their 6 months postoperative plasma TIMP-1 level and serum CEA level. Group 1 ("0 0"): low TIMP-1 and low CEA; Group 2 ("1 0"): high TIMP-1 and low CEA; Group 3 ("0 1"): low TIMP-1 and high CEA; Group 4 ("1 1"): high TIMP-1 and high CEA. The patients were dichotomised using the 95th percentile of plasma TIMP-1 levels in healthy blood donors and 5 ng/ml of CEA.

Figure 19 (A-D): Longitudinal TIMP-1 and CEA measurements in patients with prior intended radical colorectal cancer surgery. The first blood sample has been taken before operation of the cancer. The four different plots are representative for the four different scenarios. In the line above the curves the status for the patients included in the figures is indicated, LR means local recidiv, DM means distant metastasis and mors means dead – all followed by an indication of time in months if relevant.

Fig 19 A shows patients with concomitant elevation in plasma TIMP-1 and serum CEA in relation to recurrent disease.
Fig 19 B shows patients with elevation in serum CEA but not plasma TIMP-1 in relation to disease recurrence.
Fig 19 C shows patients with elevation in plasma TIMP-1 but not serum CEA in relation to disease recurrence.
Fig 19 D shows patients with continuous low serum CEA and plasma TIMP-1 and never developed disease recurrence.

EXAMPLES

Example 1

Preparation of an ELISA to quantitate total TIMP-1 concentrations in human plasma.

This example describes the preparation and validation of an ELISA that measures total TIMP-1 levels in plasma. In addition, this example provides information on TIMP-1 levels in different plasma preparations as well as in healthy blood donors of both sexes.

Materials and methods:

Blood donors

Blood samples were initially obtained from 94 apparently healthy volunteer blood donors, comprising 51 males aged 19 to 59 years (median: 41 years) and 43 females aged 20 to 64 years (median: 36 years). In a subsequent collection, 100 donor samples were obtained, comprising 56 males aged 19 to 59 years (median 42: years) and 44 females aged 20 to 60 years (median: 36.5 years). Informed consent was obtained from all donors, and permission was obtained from the local Ethical Committees.

Blood collections and plasma separation

Peripheral blood was drawn with minimal stasis (if necessary a maximum of 2 min stasis with a tourniquet at maximum +2 kPa was acceptable) into pre-chilled citrate, EDTA, or heparin collection tubes (Becton-Dickinson, Mountain View, CA), mixed 5 times by inversion, and immediately chilled on ice. As soon as possible (no later than 1.5 h after collection) the plasma and blood cells were separated by centrifugation at 4°C at 1,200 x g for 30 min, and stored frozen at -80°C prior to assay. Plasma pools were made with freshly collected samples from at least ten donors, aliquoted and stored frozen at -80°C. For analysis, the samples were quickly thawed in a 37°C water bath at and then placed on ice until needed.

TOTAL TIMP-1 ELISA

A sensitive and specific sandwich ELISA was prepared, using TIMP-1 antibodies developed at the Strangeways Laboratories (Hembry *et al*, 1985). A sheep polyclonal anti-TIMP-1 antiserum (Hembry *et al*, 1985; Murphy *et al*, 1991) was used for antigen capture, and a murine monoclonal anti-TIMP-1 IgG1 (MAC-15) (Cooksley *et al*, 1990) for antigen detection. A rabbit anti-mouse immunoglobulin/alkaline phosphatase conjugate (Catalog number D0314, Dako, Glostrup, Denmark) was the secondary detection reagent. The latter conjugate was supplied preabsorbed against human IgG, thus eliminating cross-reactivity with IgG in the plasma samples. As the monoclonal detection antibody MAC-15 recognises both free TIMP-1 and TIMP-1 in complex with MMP's (Cooksley *et al*, 1990), the total TIMP-1 content captured by the sheep polyclonal anti-TIMP-1 antiserum was quantitated by the ELISA.

96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated for 1 h at 37°C with 100 µL/well of polyclonal sheep anti-TIMP-1 (4 mg/L) in 0.1 mol/L carbonate buffer, pH 9.5. The wells were then rinsed twice with 200 µL/well of SuperBlockJ solution (Pierce Chemicals, Rockford, IL) diluted 1:1 with phosphate-buffered saline (PBS). The microtiter plates were stored for up to 14 days at -20°C. On the day of analysis, the plates were thawed at room temperature and washed 5 times in PBS containing 1 g/L Tween.

A series of purified, recombinant human TIMP-1 standards were used to calibrate each plate. Standards were prepared by serially diluting a stock solution of purified TIMP-1. Standard concentrations were 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µg/L. Included on each plate was a blank containing only sample dilution buffer, and 2 controls made from a 1:100 dilution of a citrate plasma pool. One control was added as the first sample on the plate and the second control was added as the last. All plasma samples were diluted 1:100 in sample buffer consisting of 50 mol/L phosphate, pH 7.2, 0.1 mol/L NaCl, 10 g/L bovine serum albumin (Fraction V, Boehringer-Mannheim, Penzberg, Germany), and 1 g/L Tween 20. A total of 100 µL/well of each standard, blank, control, and patient sample was incubated on the plate for 1 h at 30°C. All standards, blanks, controls, and samples were run in triplicate on each plate for every assay. After primary incubation, the wells were washed 5 times, then treated for 1 h at 30°C with 100 µL/well of purified MAC-15 monoclonal antibody (0.5 mg/L) in sample dilution buffer. After another 5 washes the wells were incubated for 1 h at 30°C with 100 µL/well of rabbit anti-mouse immunoglobulins(Ig)/alkaline phosphatase conjugate diluted 1:2000 in sample dilution buffer. Following 5 washes with washing solution and 3 washes with distilled water, 100 µL of freshly made p-nitrophenyl phosphate (Sigma, St. Louis, MO) substrate solution (1.7 g/L in 0.1 mol/L Tris.HCl, pH 9.5, 0.1 mol/L NaCl, 5 mmol/L $MgCl_2$) was added to each well. The plate was placed in a Ceres 900J plate reader (Bio-Tek Instruments, Winooski, VT) at 23°C with the yellow color development automatically monitored. Readings were taken at 405 nm against an air blank every 10 min. for one hour. KinetiCalc II software was used to analyze the data by calculating the rate of color formation for each well (linear regression analysis), generating a 4-parameter fitted standard curve, and calculating the TIMP-1 concentration of each plasma sample.

Recovery experiments

The recovery of TIMP-1 signal was measured following addition to 1:100 dilutions of citrate, EDTA or heparin plasma pools. Purified TIMP-1 was added to plasma pools to give final concentrations in the range of 0 to 10 µg/L. The recovery in each case was calculated from the slope of the line representing TIMP-1 signal as a function of concentration, where

100% recovery was defined as the slope obtained when TIMP-1 was diluted in sample dilution buffer.

Immunoblotting

Citrate plasma from a patient with a high level of TIMP-1 in blood (634 µg/L, determined by ELISA), was diluted 1:10 and added to a protein A-Sepharose column pre-incubated with polyclonal sheep anti-TIMP-1. Following 5 cycles, bound proteins were eluted from the column and 50 µL of the resulting eluate run on 12% SDS-gel electrophoresis (Ready GelJ, Bio-Rad). A mixture of low molecular weight (Pharmacia) and high molecular weight markers (Bio-Rad) and 50 µL of TIMP-1 standard (100 µg/L) in Laemmli Sample Buffer were also run on the gel. Proteins were transferred electrophoretically from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated for 1 h at room temperature with 1% skim milk powder in TBS. Following washing, the membrane was incubated for 1 h at room temperature with 20 ml of MAC-15 (5 mg/L). The membrane was then washed and incubated for an additional hour at room temperature with 20 ml of rabbit anti-mouse Ig/alkaline phosphatase conjugate diluted 1:1000. Finally the membrane was washed and color developed by the addition of a phosphate substrate solution (NBT/BCIP).

Results:

ELISA performance

Development of color in each well progressed as a linear function of time for all concentrations of total TIMP-1 in these experiments (Figure 1), with correlation coefficients for the fitted lines typically greater than 0.99. The standard curve for the rates plotted against the TIMP-1 concentration consisted of the linear and upper curved regions (over the range of 0 to 5 µg/L) of a sigmoidal curve, and the correlation coefficient for the 4-parameter fit was typically better than 0.999 (Figure 2). The rate with no TIMP-1 (read against an air blank) was 1.21±0.15 (mean±SD) milliabsorbance units/min (n=29), while the rate with 10 µg/L standard TIMP-1 was 50.3±6.01 milliabsorbance units/min (n=29). The limit of detection for the assay, defined as the concentration of TIMP-1 corresponding to a signal 3 SD above the mean for the TIMP-1 blank, was 0.089 µg/L. This value was 13% of the mean of the measured concentrations of TIMP-1 in healthy citrate plasma samples . The intra-assay coefficient of variation (CV) for 16 replicates of a control citrate plasma pool was 5.3%, and the inter-assay CV for 29 successive assays of the plasma pool (run on different days) was 6.2%. This plasma pool had a TIMP-1 concentration of 57.8 µg/L, corresponding to the 22nd centile of the normal individuals.

Recovery of recombinant TIMP-1 after dilution in plasma Specific recovery was determined by addition of increasing concentrations of purified TIMP-1 to a panel of plasma pool replicates, followed by subsequent measurement of signal. Recovery was 104% in citrate plasma, 101% in diluted EDTA plasma, and 87% in diluted heparin plasma (Figure 3). Thus the recovery of TIMP-1 signal from an internal standard was acceptable for all preparations of plasma

Dilution curves for total plasma TIMP-1 signal

Serial dilutions of citrate, EDTA and heparin plasma pools were made to test for linear reduction in TIMP-1 signal. Citrate, EDTA and heparin plasmas all gave good linearity of signal as a function of dilution. The 1% plasma dilution which was chosen for subsequent determinations lay well within the range of this linear dilution curve.

Immunoblotting of total plasma TIMP-1

A Western blot of an immunoabsorbed patient plasma sample showed a clear band of 28 kDa (Figure 4, lane 2), corresponding to free, uncomplexed TIMP-1 (Figure 4, lane 1). No bands were found at the expected higher molecular weights corresponding to complexes between MMP's and TIMP-1, e.g. MMP-2:TIMP-1, 100 kDa. This indicates either that the major part of TIMP-1 was present in the plasma as the free form, or that complexes were dissociated during SDS-PAGE. Although the sample was left both unreduced and unheated in order to preserve any complexes present in the plasma sample, it has been reported that MMP:TIMP complexes may be unstable in SDS-PAGE (Wilhelm *et al*, 1989; Stetler-Stevenson *et al*, 1989; Moll *et al*, 1990), even under non-reducing conditions (Moutsiakis *et al*, 1992).

Total TIMP-1 in citrate and EDTA plasma from the same healthy donor

A collection of citrate and EDTA plasma samples taken simultaneously from 100 healthy donors was available for this study. These samples were not specifically collected as platelet-poor plasma. However, a small, representative number of samples, prepared as platelet-poor plasma, did not differ significantly in total TIMP-1 values. The percentile plots for total TIMP-1 levels in these samples are shown in Figure 5a. The values in each set approximated a normal distribution. Citrate plasma TIMP-1 levels ranged between 55.0 and 90.3 μg/L (10th to 90th percentile) with a mean of 69.2±13.1 μg/L. Similarly, EDTA plasma TIMP-1 levels ranged from 58.0 to 91.8 μg/L with a mean of 73.5±14.2 μg/L. For both citrate and EDTA plasma, the mean TIMP-1 levels were in close proximity to the median levels (Table 1). A paired means comparison showed that the level of TIMP-1 in citrate

plasma was significantly lower by 4.34 μg/L (95% CI 2.34-6.33; ($p<0.0001$) than the EDTA plasma level from the same individual. However, it is likely that this difference may be due to the variability in sampling procedure during plasma collection. EDTA plasma tubes contained dry anticoagulant material, while citrate plasma tubes contained a small amount of liquid citrate buffer which gave a small and variable systematic dilution error (x 9/10). The level of TIMP-1 in citrate plasma correlated with that in EDTA plasma from the same individuals. The linear regression plot in Figure 5b shows a regression coefficient of 0.99 with a slope of the fitted line of 0.93, perhaps illustrating this small dilution error. A non-parametric Spearman's rank test for the data set gave a rho value of 0.62 and $p<0.0001$.

Total TIMP-1 levels in citrate plasma

A total of 194 citrate plasma samples from healthy blood donors were assayed, comprising 94 samples taken during one collection and 100 samples taken 9 months later from a different set of donors. Figure 6 shows the percentile plots for TIMP-1 levels measured in these two independent groups. The reference range for TIMP-1 levels in citrate plasma from the first collection was 53.3 to 77.7 μg/L (10th to 90th percentile) with a mean of 65.4±10.1 μg/L which was indistinguishable from the median (Table 1). and approximating a normal distribution. The mean TIMP-1 level for the second collection was 69.2±13.1 μg/L (reference range 55.0 to 90.3 μg/L). An unpaired means comparison showed that TIMP-1 levels in the two sets of samples taken during two different periods differed only by 3.82 μg/L (95% CI: 0.50-7.14 μg/L; $p=0.024$). Moreover, no significant difference was apparent between the controls ($n=8$) included in each set of assays (mean difference 0.36 μg/L; 95% CI: 1.71-2.44 μg/L; $p=0.69$). The mean TIMP-1 level for all 194 citrate plasma samples was 67.3±11.8 μg/L, close to the median of 66.1 μg/L, with levels again approximating a normal distribution (reference range 54.0 to 82.7 μg/L).

TABLE 1:

SUMMARY OF TOTAL TIMP-1 LEVELS DETERMINED IN BLOOD FROM HEALTHY DONORS:

Blood fraction	Date of sampling	Number of samples	Mean±SD (µg/L)	Median (µg/L)	Reference range* (µg/L)
Citrate plasma	Sept. 96	94	65.4±10.1	65.6	53.3-77.7
Citrate plasma	May 97	100**	69.2±13.1	67.0	55.0-90.3
Citrate plasma	96+97	194	67.3±11.8	66.1	54.0-82.7
EDTA plasma	May 97	100**	73.5±14.2	71.2	58.0-91.8

*The reference range is defined as the 10th to 90th percentile.

**These samples were collected from the same donors.

In figure 8 the plasma TIMP-1 levels are shown for 807 health blood donors.

Tests for correlations to gender and age of the donor

In these studies, the control values measured in each assay had a CV of 2.7%. Percentiles for TIMP-1 levels in 194 citrate plasma samples calculated according to gender are shown in Figure 7. The mean TIMP-1 value for 107 male donors was 70.4±12.0 µg/L (median 69.4 µg/L) with a reference range from 56.2 to 86.6 µg/L, while the mean TIMP-1 value for 87 female donors in this set was 63.5±10.5 µg/L (median: 62.0 µg/L) with a reference range from 51.8 to 77.0 µg/L. There was a significant difference ($p<0.0001$) in TIMP-1 mean levels between the two groups, with males having higher TIMP-1 levels than females. There was a trend towards an increase in plasma TIMP-1 with increasing age (Spearman's rho=0.33, P=0.0011), but this did not increase with gender (females: Spearman's rho=0.29, P=0.006; males: Spearman's rho=0.35, P=0.0003). In EDTA plasma, the mean TIMP-1 value for 56 males was 76.9±15.0 µg/L (median: 75.1 µg/L) with a range from 58.8 to 96.9 µg/L, while 44 female donors had a mean TIMP-1 level of 69.3±11.8 µg/L (median: 67.9) with a reference range from 56.1 to 85.5 µg/L. Again, a significant difference appeared between males and females ($p=0.0076$, unpaired means comparison).

Discussion:

The assay described above enables accurate determination of total TIMP-1 in human plasma samples. Kinetic rate assays of the bound antigen were easily accomplished, per-

mitting automated fitting of rate curves, which has proven considerably more reliable than single end-point measurements. The use of a rapid blocking agent and a dilution buffer with high buffering capacity also contributed to reproducible assays. Incorporating all these elements in the final assay fulfilled the requirements of sensitivity, specificity, stability, and good recovery of an internal standard.

The quantitative studies in blood from healthy donors showed that both citrate and EDTA plasma samples are suitable for TIMP-1 determination. Compared to other published studies of TIMP-1 i from healthy donors (Jung *et al*, 1996; Jung *et al*, 1996), levels in the present study fell within a very narrow range. Some studies have reported results in serum, but plasma was selected for the present study to avoid the variable contribution of platelet activation to the measured TIMP-1 values (Cooper *et al*, 1985). While the plasma samples used in this study were not specifically prepared as platelet-poor plasma, it was shown, based on tests carried out in the lab, that this does not change the values. The donor material was large enough to demonstrate that TIMP-1 levels in healthy individuals (both EDTA and citrate) approximated a normal distribution, for females as well as for males. Mean TIMP-1 levels were approx. 10% higher in males than in females for both EDTA and citrate plasma. One explanation for this is a higher release rate of TIMP-1 into blood from activated platelets, reflecting a tendency towards higher incidence of thromboembolic disease in the male population. When males and females were considered separately, there was a weak correlation between TIMP-1 and age as seen for the whole population (see above).

Example 2

Preparation of an ELISA to quantitate TIMP-1:MMP-9 complexes in plasma.

The following example describes an assay to determine the concentration of TIMP-1:MMP-9 complexes in body fluids. The assay is used with plasma samples of healthy blood donors in order to establish normal ranges of this complex (Holten Andersen et al., 1999).

Materials and methods:

TIMP-1:MMP-9 complex ELISA

A sensitive and specific sandwich ELISA was prepared using the above-described TIMP-1 antibody, MAC-15, and a rabbit MMP-9 polyclonal antibody developed in the Hematological Department, Rigshospitalet, Denmark (Kjeldsen *et al*, 1992). The MMP-9 antibody was used for antigen capture and MAC-15was used for antigen detection. A rabbit anti-mouse-Ig/alkaline phosphatase conjugate (Dako, Glostrup, Denmark) enabled a kinetic rate assay

(Figure 9). The latter conjugate was supplied preabsorbed against human IgG, thus eliminating cross-reactivity with IgG in the plasma samples. The MMP-9 antibody captured both free MMP-9 and MMP-9 complexed with TIMP-1, while MAC-15 only recognised TIMP-1. Therefore only TIMP-1:MMP-9 complexes were quantitated by this reagent pair.

To prevent spontaneous, *ex vivo* TIMP-1:MMP complex formation during sampling and assay procedures, a protease inhibitor (ie. Galardin, Batimastat, Marimastat) was added to the plasma sample after thawing. The addition of the protease inhibitor blocked *in vitro* complex formation by inhibition of the catalytic activity of the metalloproteinases.

The TIMP-1:MMP-9 assay was prepared and validated by a method similar to that described above for the total TIMP-1 assay. The TIMP-1:MMP-9 standard was prepared by incubating equimolar amounts of purified recombinant TIMP-1 and MMP-9 (activated by adding APMA) in PBS for 1 hour at 37 degrees Celsius.

Briefly, 96-well micotiter plates were coated overnight at 4°C with 100 µL/well of rabbit polyclonal anti-MMP-9 antiserum in 0.1 mol/L carbonate buffer, pH 9.5. Prior to use, assay wells were rinsed twice with 200 µL/well of SuperBlock solution diluted 1:1 with phosphate-buffered saline (PBS), and then washed 5 times in PBS containing 1 g/L Tween 20. Wells were then incubated for 1 h at 30°C with 100 µL/well of plasma diluted in sample buffer A series of purified TIMP-1:MMP-9 standards were used to calibrate each plate. Standards were prepared by serially diluting a stock solution of purified TIMP-1:MMP-9 complex. Included on each plate was a blank containing only sample dilution buffer, and 2 controls made from a citrate plasma pool. One control plasma pool was added as the first sample on the plate and the second control was added as the last. All standards, blanks, controls, and samples were run in triplicate on each plate for every assay. After sample incubation and TIMP-1:MMP-9 complex binding, the wells were washed 5 times, followed by treatment for 1 h at 30°C with 100 µL/well of MAC-15 in sample dilution buffer. After another 5 washes, the wells were incubated for 1 h at 30°C with 100 µL/well of rabbit anti-mouse Ig/alkaline phosphatase conjugate diluted in sample dilution buffer. Following 5 washes with washing solution and 3 additional washes with distilled water, 100 µL of freshly made p-nitrophenyl phosphate substrate solution was added to each well. The plate was placed in a Ceres 900J plate reader at 23°C with the yellow color development monitored automatically. Readings were taken at 405 nm against an air blank every 10 min for one hour. KinetiCalc II software was used to analyze the data by calculating the rate of color formation for each well (linear regression analysis), generating a 4-parameter fitted standard curve, and calculating the TIMP-1:MMP-9 concentration of each plasma sample.

Recovery experiments

Specific recovery was determined by addition of TIMP-1:MMP-9 complex to a series of citrate, EDTA or heparin plasma pools. The recovery in each case was calculated from the slope of the line representing TIMP-1:MMP-9 complex signal as a function of concentration, where 100% recovery was defined as the slope obtained when TIMP-1:MMP-9 complex was diluted in the sample dilution buffer.

Results

ELISA performance

Development of color in each well was a linear function of time for all concentrations of TIMP-1:MMP-9 complexes measured in these experiments, with correlation coefficients for the automatically fitted lines typically greater than 0.9. The correlation coefficient for the 4-parameter fit was typically greater than 0.999.

Recovery of TIMP-1:MMP-9 complex after dilution in plasma

Specific recovery was determined by addition of increasing concentrations of TIMP-1:MMP-9 to a plasma pool and subsequent measurement of the specific signal.

Dilution curves for plasma TIMP-1:MMP-9

Serial dilutions of citrate and EDTA plasma pools were made and complex levels quantitated to determine the linearity of the assay. Citrate and EDTA plasmas all gave good linearity of signal as a function of dilution.

Example 3

Preparation of an ELISA to quantitate free TIMP-1 levels in plasma.

The following example describes an assay that determines the concentration of free TIMP-1 levels in body fluids. The assay is applied to plasma samples of healthy blood donors in order to establish normal ranges of free TIMP-1.

Materials and methods:

FREE TIMP-1 ELISA

A sensitive and specific sandwich immunoassay was prepared, using a TIMP-1 monoclonal IgG1 antibody (MAC-19) developed at the Strangeways Laboratories, England (Cooksley *et al*, 1990) and a sheep polyclonal anti-TIMP-1 antibody. The sheep polyclonal anti-TIMP-1 antibody was used for antigen capture and the murine monoclonal MAC-19 was used for antigen detection. A rabbit anti-mouse-Ig/alkaline phosphatase conjugate was the secondary detection reagent (Figure 10). The latter conjugate was supplied preabsorbed against human IgG, thus eliminating cross-reactivity with IgG in the plasma samples. The MAC-19 monoclonal antibody is completely specific for free TIMP-1, which therefore is the only form quantitated in this assay.

In order to test that MAC19 does not react with complexes between TIMP-1 and MMP-9, the rabbit polyclonal anti-MMP-9 antibody described in Example 2 was used for antigen capture and the mouse monoclonal antibody MAC19 for antigen detection. A rabbit anti-mouse-Ig/alkaline phosphatase conjugate was used as the secondary labelled reagent. Standard TIMP-1:MMP-9 complex, free MMP-9, free TIMP-1, and a blank control were assayed. Figure 11 shows that TIMP-1:MMP-9 complexes bound by the polyclonal anti-MMP-9 antibody are not detected by MAC19. An equivalent experiment was performed, where MAC19 was substituted with MAC15. Figure 12 shows the results of this experiment. It is seen that MAC15 detects TIMP-1:MMP-9 complex bound by the polyclonal anti-MMP-9 antibody.

To prevent *ex vivo* formation of TIMP-1:MMP complexes during the sampling and assay procedures, a protease inhibitor (ie. Galardin, Batimastat, Marimastat) can be added to the plasma sample after thawing. The addition of the protease inhibitor will prevent *in vitro* complex formation by competition with the TIMP.

96-well micortiter plates were coated for 1 h at 37°C with 100 µL/well of polyclonal sheep anti-TIMP-1 (4 mg/L) in 0.1 mol/L carbonate buffer, pH 9.5. The assay wells were then rinsed twice with 200 µL/well of SuperBlock solution diluted 1:1 with phosphate-buffered saline (PBS). The microtiter plates were stored for up to 14 days at -20°C. On the day of use, the plates were thawed at room temperature and washed 5 times in PBS containing 1 g/L Tween 20. Wells were then incubated for 1 h at 30°C with 100 µL/well of triplicate 1:25 dilutions of plasma made in a sample buffer consisting of 50 mol/L phosphate, pH 7.2, 0.1 mol/L NaCl, 10 g/L bovine serum albumin (Fraction V, Boehringer-Mannheim, Penzberg, Germany) and 1 g/L Tween 20. Standards were prepared by serially diluting a stock solution of purified free TIMP-1 to yield concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313 and

0.156 µg/L. Included on each plate was a blank containing only sample dilution buffer, and 2 controls made from a citrate plasma pool diluted 1:25. One control plasma pool was added as the first sample on the plate and the second control was added as the last. All standards, blanks, controls, and samples were run in triplicate on each plate for every assay. After TIMP-1 binding, the wells were washed 5 times, then treated for 1 h at 30°C with 100 µL/well of the purified murine monoclonal anti-TIMP MAC-19 (0.5 mg/L) in sample dilution buffer. After another 5 washes the wells were incubated for 1 h at 30°C with 100 µL/well of rabbit anti-mouse immunoglobulins/alkaline phosphatase conjugate diluted 1:2000 in sample dilution buffer. Following 5 washes with washing solution and 3 washes with distilled water, 100 µL of freshly made p-nitrophenyl phosphate (Sigma, St. Louis, MO) substrate solution (1.7 g/L in 0.1 mol/L Tris.HCl, pH 9.5, 0.1 mol/L NaCl, 5 mmol/L $MgCl_2$) was added to each well, and the plate was placed in a Ceres 900J plate reader (Bio-Tek Instruments, Winooski, VT) at 23°C. The yellow color development was monitored automatically, with readings taken at 405 nm against an air blank every 10 min for one hour. KinetiCalc II software was used to analyze the data, to calculate the rate of color change for each well (linear regression analysis), and to compute a 4-parameter fitted standard curve, from which the free TIMP-1 concentration of each plasma sample was calculated.

Dilution curves and recovery experiments

These experiments were performed essentially as described in Example 1, but using MAC19 instead of MAC15 for detection of free TIMP-1 (as described above).

Healthy donors

108 donor plasma samples were obtained. Donors gave blood on a volunteer basis and were all apparently healthy. Informed consent was obtained from all blood donors, and permission was obtained from the local Ethical Committees. The blood sampling and handling were performed as described in Example 1.

Results

ELISA performance

Development of color in each well was a linear function of time for all concentrations of free TIMP-1 measured in these experiments, with correlation coefficients for the automatically fitted lines typically better than 0.9. The correlation coefficient for the 4-parameter fit was typically better than 0.999. The intra-assay variation for 24 triplicate measurements of the control plasma pool was 9.6%.

Recovery of recombinant TIMP-1 after dilution in plasma

Specific signal recovery was determined by addition of increasing concentrations of purified TIMP-1 standard to a plasma pool and subsequent measurement of the ELISA signal. In the diluted citrate plasma pool 105% recovery was obtained, while 96% recovery was obtained in the diluted EDTA plasma pool (Figure 13).

Dilution curves for free plasma TIMP-1 signal

Serial dilutions of citrate and EDTA plasma pools were made and free TIMP-1 levels assayed to test for linear reduction in ELISA signal. Citrate and EDTA plasmas all gave good linearity of signal as a function of dilution.

Healthy blood donors

Free TIMP-1 was measurable in all plasma samples. The median free TIMP-1 concentration was 70.9 µg/L (range: 32.3-169.7 µg/L).

Discussion

This assay directly measures plasma free TIMP-1 levels. When comparing free TIMP-1 levels with total TIMP-1 levels (the latter measured with assay described in Example 1) in the 108 healthy blood donors, a correlation coefficien of 0.46 (Rho, Spearman Rank, $p<0.0001$) was obtained.

Example 4

Total TIMP-1 as obtained 6 months post-operatively in patients with a prior surgery for colorectal cancer.

Total TIMP-1 levels in plasma obtained 6 months post-operatively from 272 colorectal cancer patients and in plasma from 807 individuals were measured with the TIMP-1 assay described in Example 1. The TIMP-1 values were analyzed and compared using standard biostatistical parameters.

Materials and methods:

Patients

293 colorectal cancer patients were included in the study. 44 patients had Dukes' A disease, 137 Dukes' B, 112 Dukes' C. All patients received intended curative surgery but no adjuvant chemotherapy was administered. Corresponding plasma and serum samples were obtained preoperatively and 6 months postoperatively with informed consent from all patients in accordance with the Helsinki declaration, and permission was granted by the local ethical committee of Hvidovre Hospital, Denmark. The median follow-up time was 82 months (range 68 to 95); 141 patients died, 65 patients experienced local recurrence and 49 patients developed distant metastases during the observation period.

Healthy donors

Plasma samples from 807 healthy blood donors were collected. 417 were females and 390 were males. The median age was 42 years and 25% of the donors were above 53 years of age. The youngest donor was 18 years of age..

Blood samples

Blood samples (5 ml) were collected post-operatively from all patients. To ensure valid TIMP-1 measurements, peripheral blood was drawn with minimal stasis and collected in EDTA anticoagulant tubes (Becton-Dickinson, Mountain View, CA) in accordance with a previously described protocol (Example 1).

TIMP-1 ELISA

TIMP-1 levels were measured in EDTA plasma samples using the assay described in Example 1.

Results:

Total TIMP-1 levels in plasma

Using a kinetic rate assay, total TIMP-1 levels were determined in all patient and healthy donor plasma samples. Every plasma sample had measurable levels of TIMP-1.

The median TIMP-1 level in plasma from healthy donors was 72.3 µg/L with a range of 30.1 - 228.6 µg/L. There was a highly statistical difference in the total plasma TIMP-1 values between the colorectal cancer patients and the healthy blood donors Figure 14.

The 95th percentile of the healthy blood donors was 112.1 µg/L plasma.

Example 5

Diagnostic value of total TIMP-1

Total TIMP-1 levels in plasma obtained preoperatively and 6 months post-operatively from 309 colorectal cancer patients with Dukes A+B cancer or Dukes C cancer. Plasma TIMP-1 was measured in samples obtained preoperatively and 6 months post-operatively. Plasma TIMP-1 was measured with the TIMP-1 assay described in Example 1. The TIMP-1 values were analyzed and compared using standard biostatistical parameters.

Materials and methods:

Patients

309 colorectal cancer patients were included in the study. 48 patients had Dukes' A disease, 141 Dukes' B, and 120 Dukes' C. All patients received intended curative surgery but no adjuvant chemotherapy was administered. Corresponding plasma and serum samples were obtained preoperatively and 6 months postoperatively with informed consent from all patients in accordance with the Helsinki declaration, and permission was granted by the local ethical committee of Hvidovre Hospital, Denmark. The median follow-up time was 82 months (range 68 to 95)

Blood samples

Blood samples (5 ml) were collected post-operatively from all patients. To ensure valid TIMP-1 measurements, peripheral blood was drawn with minimal stasis and collected in EDTA anticoagulant tubes (Becton-Dickinson, Mountain View, CA) in accordance with a previously described protocol (Example 1).

TIMP-1 ELISA

TIMP-1 levels were measured in EDTA plasma samples using the assay described in Example 1. Plasma TIMP-1 levels were scored as low or high based on the 95th percentile of plasma TIMP-1 in the 807 healthy blood donors.

Results

Preoperative and postoperative plasma TIMP-1 levels were scored as low or high based on the 95th percentile of plasma TIMP-1 in the 807 healthy blood donors. 82% (114/139) of

the patients with low preoperative TIMP-1 levels remained low postoperatively (group I) and accordingly 18% (25/139) progressed to high postoperative TIMP-1 levels (group III). 38% (58/154) of the patients with high preoperative levels had low postoperative TIMP-1 (group II) and accordingly 62.3% (96/154) remained high 6 months following surgery (group IV). Analysis of overall survival showed a significant difference between groups ($p<0.0001$). With group I as the baseline, group II had a hazard ratio (HR) of 1.1 (95% CI: 0.7 to 1.8), group III 1.3(0.7 to 2.4) and group IV 1.6 (1.1 to 2.4). Multivariate analysis including Dukes' stage, age, gender, tumor location, serum CEA and plasma TIMP-1 (as grouped into the above mentioned four groups), showed that plasma TIMP-1 was a significant and independent ($p=0.0004$) predictor of survival. Similar results were obtained for time to local recurrence and detection of distant metastases. Separate analyses including only the 6 month value of TIMP-1 indicated that elevated levels were associated with shorter survival ($p<0.0001$) (Figure 15).

Discussion:

These data suggest that comparing preoperative and postoperative total TIMP-1 measurements in plasma can be used as a monitoring procedure to aid in identifying patients with a high risk of having MRD and/ or recurrent colorectal cancer.

Example 6

Diagnostic value of total TIMP-1 as obtained 6 months post-operatively in patients with a prior surgery for Dukes A + B or C colorectal cancer.

Total TIMP-1 levels in plasma obtained 6 months post-operatively from 309 colorectal cancer patients with Dukes A+B cancer or Dukes C cancer. Plasma TIMP-1 was measured in samples obtained 6 months post-operatively. Plasma TIMP-1 was measured with the TIMP-1 assay described in Example 1. The TIMP-1 values were analyzed and compared using standard biostatistical parameters.

Materials and methods:

Patients

309 colorectal cancer patients were included in the study. 48 patients had Dukes' A disease, 141 Dukes' B, and 120 Dukes' C. All patients received intended curative surgery but no adjuvant chemotherapy was administered. Corresponding plasma and serum samples were obtained 6 months postoperatively with informed consent from all patients in accor-

dance with the Helsinki declaration, and permission was granted by the local ethical committee of Hvidovre Hospital, Denmark. The median follow-up time was 82 months (range 68 to 95)

Blood samples

Blood samples (5 ml) were collected post-operatively from all patients. To ensure valid TIMP-1 measurements, peripheral blood was drawn with minimal stasis and collected in EDTA anticoagulant tubes (Becton-Dickinson, Mountain View, CA) in accordance with a previously described protocol (Example 1).

TIMP-1 ELISA

TIMP-1 levels were measured in EDTA plasma samples using the assay described in Example 1. Postoperative plasma TIMP-1 levels were scored as low or high based on the 95th percentile of plasma TIMP-1 in an age matched healthy control group.

Results:

Total TIMP-1 levels in plasma

Using a kinetic rate assay, total TIMP-1 levels were determined in all patient plasma samples. Every plasma sample had measurable levels of TIMP-1.

Diagnostic value of total TIMP-1

Using the measured total TIMP-1 levels in plasma from the 309 colorectal cancer patients, univariate survival analyses was performed. As seen in Figure 16, Dukes A+B patients with low post-operative plasma TIMP-1 levels had a significantly better survival than Dukes A+B patients with high post-operative plasma TIMP-1 levels. Similar results were obtained in patients who prior to the blood sampling underwent surgery for Dukes C disease. It is noteworthy, that Dukes A+B patients with high post-operative plasma TIMP-1 levels had a survival similar to the survival of Dukes C patients with low post-operative TIMP-1 plasma levels.

Discussion:

These data suggest that total post-operative TIMP-1 measurements in plasma can be used as a monitoring procedure to aid in identifying Dukes A, B and C patients with a high risk of having MRD and/ or recurrent colorectal cancer.

Example 7

Diagnostic value of total TIMP-1 in combination with CEA in patients with a prior surgery for colorectal cancer.

Total TIMP-1 in plasma from 309 colorectal cancer patients was measured using the TIMP-1 assay described in Example 1. In addition, CEA was measured in the corresponding patient serum samples using a commercially available, chemiluminescent CEA EIA kit (Immulite CEA, DPC™, Los Angeles, California, USA). The TIMP-1 and CEA values from the cancer patients were used to construct univariate survival curves (metastasis free survival (MFS) and overall survival (OS)). The patients were dichotomised using the 95th percentile plasma TIMP-1 level of a healthy blood donor population.

Results:

Diagnostic value of total TIMP-1 and CEA

As seen from Figure 17 and Figure 18, patients with low TIMP-1 and low CEA levels had a MFS and OS which was significantly better than those of patients with one or both parameters being high. On the other hand, patients with both parameters high had a MFS and OS which were significantly worse than those of patients with one or none of the parameters being high (Figure 17 and Figure 18).

Discussion:

These data show that by adding an additional marker (CEA), an improvement in the monitoring value of total TIMP-1 can be obtained. Thus, the combination of CEA and TIMP-1 could be useful as a monitoring procedure to identify patients with a high risk of having MRD or recurrent colorectal cancer. It should be noted that this combination was efficient in identifying patients with early stage cancer (Duke's stage A+B) with a high probability of MRD and/or recurrent colorectal cancer.

Example 8

Quantitation of total TIMP-1 in plasma from patients with inflammatory bowel diseases.

Patients

46 patients with IBD (Inflammatory Bowel Disease) were included in the study. 22 patients had ulcerative colitis and 24 patients had Crohn's Disease. Total TIMP-1 levels in EDTA plasma from healthy blood donors and post-operatively in colorectal cancer patients (Examples 4) were included for comparison.

TOTAL TIMP-1 VALUES

Total TIMP-1 levels were measured in the EDTA plasma samples using the sandwich assay described in Example 1.

Results:

The measured total TIMP-1 values are shown in Table 2.

Table 2

	Ulcerative colitis (n=22)	Crohn's disease (n=24)	IBD total (n=46)
Median total TIMP-1 (µg/L)	79.5	78.3	84.8
Range total TIMP-1 (µg/L)	54.9-189.9	49.0-156.2	38.7-154.5

	Healthy donors (n=108)	Colorectal Cancer (n=315)
Median total TIMP-1 (µg/L)	89.1	105
Range total TIMP-1 (µg/L)	51.0-156.2	44-450

There was no significant difference when total TIMP-1 values from patients with IBD and healthy blood donors were compared (Mann-Whitney; p=0.45). There was a highly significant difference between total plasma TIMP-1 levels between patients with IBD and the 315 colorectal cancer patients (Mann-Whitney; p<0.0001).

Discussion:

These results demonstrate that patients with colorectal cancer have significantly higher total TIMP-1 plasma levels than do patients with IBD. Moreover, patients with IBD had total TIMP-1 levels equivalent to those found in healthy blood donors, showing that plasma

total TIMP-1 can be used as a highly sensitive and specific marker to distinguish between non-malignant and malignant diseases of the gastrointestinal tract.

Example 9

Longitudinal measurement of plasma TIMP-1 and serum CEA in patients with a prior colorectal cancer:

Patients:

260 patients with prior colorectal cancer surgery were included. The TIMP-1 assay described in Example 1 was used to measure plasma TIMP-1 levels. In addition, CEA was measured in the corresponding patient serum samples using a commercially available, chemiluminescent CEA EIA kit (Immulite CEA, DPCTM, Los Angeles, California, USA).

Blood samples:

Plasma and serum samples were obtained at intervals after the primary surgery.

Results:

As seen in Figure 19, which is representative for the data obtained, some patients had concomitant elevation in plasma TIMP-1 and serum CEA in relation to recurrent disease (A), some had elevation in serum CEA but not plasma TIMP-1 in relation to disease recurrence (B), some had elevation in plasma TIMP-1 but not serum CEA in relation to disease recurrence (C) and some had continuous low serum CEA and plasma TIMP-1 and never developed disease recurrence (D).

Discussion:

These data suggest that serum CEA and plasma TIMP-1 is additive in detecting recurrent disease in patients with prior surgery for colorectal cancer.

Example 10

Monitoring of colorectal cancer patients during adjuvant systemic anti-neoplastic treatment

Patients. Colorectal cancer patients who receive adjuvant systemic treatment for primary colorectal cancer. Plasma TIMP-1 concentrations are measured by the TIMP-1 assay

described in Example 1. In addition, CEA is measured in the corresponding patient serum samples using a commercially available, chemiluminescent CEA EIA kit (Immulite CEA, DPCTM, Los Angeles, California, USA).

Blood sampling:

Serum and plasma samples are collected before each course of treatment.

Results:

Patients with plasma TIMP-1 levels above the 95th percentile of healthy blood donors and/or serum CEA levels above 5 ng/ml and who do not respond by a decrease in these levels upon a few number of cycles (e.g. 2 or 3) of the adjuvant treatment, are considered as non-responders and should therefore be taken off treatment and offered an alternative systemic treatment.

Discussion

This example describes a way to detect treatment failure early and thus allowing an early change in treatment modality.

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CLAIMS

1. A method for determining whether an individual is likely to have minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer after being treated for the primary cancer, the method comprising determining at least one first parameter representing a post-operative concentration of TIMP-1 in body fluid samples, and indicating the individual as having a high likelihood of having minimal residual disease recurrent cancer or a combination of minimal residual disease and recurrent cancer if the at least one first parameter is at or beyond a discriminating value and indicating the individual as unlikely of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the parameter is not at or beyond the discriminating value.

2. A method according to claim 1, wherein the discriminating value is a value which has been determined by measuring said at least one first parameter in both a healthy control population and in a population with known minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer, thereby determining the discriminating value which identifies the minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer population with a predetermined specificity or a predetermined sensitivity.

3. A method according to claim 1, wherein the discriminating value is a value which has been determined by measuring said at least one first parameter in a healthy control population, thereby determining the discriminating value which identifies individuals with increased plasma TIMP-1 values.

4. A method for determining whether an individual is likely to have minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer after being treated for the primary cancer, the method comprising determining a post-operative level of at least one first parameter representing the concentration of TIMP-1 in body fluid samples, and indicating the individual as unlikely of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the first parameter is below a discriminating value and indicating the individual as having a high likelihood of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the first parameter is not below the discriminating value.

5. A method according to claim 4, wherein the discriminating value is the pre-operative level of said at least one first parameter representing the concentration of TIMP-1 in body fluid samples.

6. A method according to claim 4 or 5, wherein the post-operative level of at least one first parameter representing the concentration of TIMP-1 in body fluid samples is below the discriminating value if the post-operative level of said first parameter is at least 0.001% below the discriminating value.

7. A method according to claim 6, wherein the post-operative level of said first parameter is obtained at any time after the operation, such as 2 weeks post-operation, 1 month post-operation, 1.5 month post-operation, 2 month post-operation, 3 month post-operation, 4 month post-operation, 5 month post-operation, 6 month post-operation, 7 month post-operation, 8 month post-operation.

8. A method of monitoring cancer patients receiving post-operation adjuvant treatment for a cancer, the method comprising determining at least one first parameter representing a concentration of TIMP-1 in body fluid samples after at least one cycle of adjuvant treatment, and indicating the individual as not responding to the adjuvant treatment if the at least one first parameter is at or beyond a post-operative concentration of TIMP-1 obtained before the first cycle of adjuvant systemic anti-neoplastic treatment and indicating the individual as responding to the adjuvant treatment if the parameter is not at or beyond the post-operative concentration of TIMP-1 obtained before the first cycle of adjuvant systemic anti-neoplastic treatment.

9. A method according to claim 8, wherein the cancer is a primary cancer.

10. A method according to any one of the preceding claims, wherein the at least one first parameter is the total concentration of TIMP-1.

11. A method according to any one of claims 1-9, wherein the at least one first parameter determined is the value obtained by combining the concentration of total TIMP-1 with the concentration of free TIMP-1.

12. A method according to any one of claims 1-9, wherein the at least one first parameter determined is the value obtained by combining the concentration of total TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP.

13. A method according to any one of claims 1-9, wherein the at least one first parameter determined is the value obtained by combining the concentration of free TIMP-1 with the concentration of a complex between TIMP-1 and a specific MMP.

14. A method according to any one of claims 8-13, wherein the combining is performed by logistic regression analysis.

15. A method according to any one of the preceding claims, which comprises additionally determining at least one second parameter, the second parameter representing the concentration of an additional marker different from any form of TIMP-1, in a body fluid sample from the individual.

16. A method according to claim 15, wherein the at least one first parameter representing the concentration of TIMP-1 in body fluid samples and the at least one second parameter different from any form of TIMP-1 are combined to result in a combined parameter and indicating the individual as having a high likelihood of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the combined parameter is at or beyond a discriminating value and indicating the individual as unlikely of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the combined parameter is not at or beyond the discriminating value.

17. A method according to claim 16, wherein the combining is performed by logistic regression analysis.

18. A method according to claim 16 or 17, wherein the discriminating value of the combined parameter is a value which has been determined by determining said combined parameter in both a healthy control population and a population with known minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer , thereby determining the discriminating value which identifies the population with minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer with a predetermined specificity or a predetermined sensitivity

19. A method according to claim 16 or 17, wherein the discriminating value of the combined parameter is a value which has been determined by measuring said combined parameter in a healthy control population, thereby determining the discriminating value which identifies individuals with increased plasma TIMP-1 values.

20. A method according claim 15, wherein the at least one first parameter representing the concentration of TIMP-1 in body fluid samples and the at least one second parameter different from any form of TIMP-1 are combined to result in a combined parameter and indicating the individual as unlikely of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the post-operative combined parameter is below a discriminating value and indicating the individual as having a high likelihood of having minimal residual disease, recurrent cancer or a combination of minimal residual disease and recurrent cancer if the post-operative value of the combined parameter is not below the discriminating value.

21. A method according to claim 20, wherein the discriminating value is the pre-operative level of said combined parameter.

22. A method according to claim 20 or 21, wherein the post-operative level of the combined parameter is below the discriminating value if the post-operative level of the combined parameter is at least 0.001% below the pre-operative level, such as at least 0.01% below, e.g. 0.1% below, e.g. 1% below the pre-operative level.

23. A method according to claim 22, wherein the post-operative level of the combined parameter is obtained after the operation, such as 2 weeks post-operation, 1 month post-operation, 1.5 month post-operation, 2 month post-operation, 3 month post-operation, 4 month post-operation, 5 month post-operation, 6 month post-operation, 7 month post-operation, 8 month post-operation.

24. A method according to claim any one of claims 20-23, wherein the combining is performed by logistic regression analysis.

25. A method according to any one of claims 15-23, wherein the at least one second parameter determined is a parameter representing the concentration of a tumour marker.

26. A method according to claim 25, wherein the tumour marker is selected from the group consisting of CEA, soluble u-PAR, any fractions of soluble u-PAR, cathepsin B, cathepsin H, HER2-neu, CA15-3 and YKL-40, 19.9 and CA242.

27. A method according to claim 26, wherein the at least one second parameter determined is the concentration of CEA.

28. A method according to any one of the preceding claims, wherein the individual is a member of a population already identified as having an increased risk of developing recurrent cancer.

29. A method according to any one of the preceding claims, wherein the determination is performed at several time points at intervals as part of a monitoring of a cancer patient during treatment for primary cancer.

30. A method according to any one of claims 1-27, wherein the determination is performed at several time points at intervals as part of a monitoring of a cancer patient after the treatment for primary cancer.

31. A method according to any one of the preceding claims, wherein the body fluid is selected from the group consisting of blood, serum, plasma, saliva, faeces, urine and cerebrospinal fluid.

32. A method according to claim 31, wherein the body fluid is plasma.

33. A method according to claim 31, wherein the body fluid is blood.

34. A method according to claim 31, wherein the body fluid is saliva.

35. A method according to claim 31, wherein the body fluid is urine.

36. A method according to any of the preceding claims, wherein the concentration determination is performed by means of an immuno assay or an activity assay.

37. A method according to claim 36, wherein the immuno assay is an ELISA.

38. A method according to claim 37, wherein the activity assay is zymography.

39. A method according to any of the preceding claims, wherein the cancer is a gastrointestinal cancer.

40. A method according to claim 39, wherein the cancer is selected from the group consisting of colon cancer and rectal cancer.

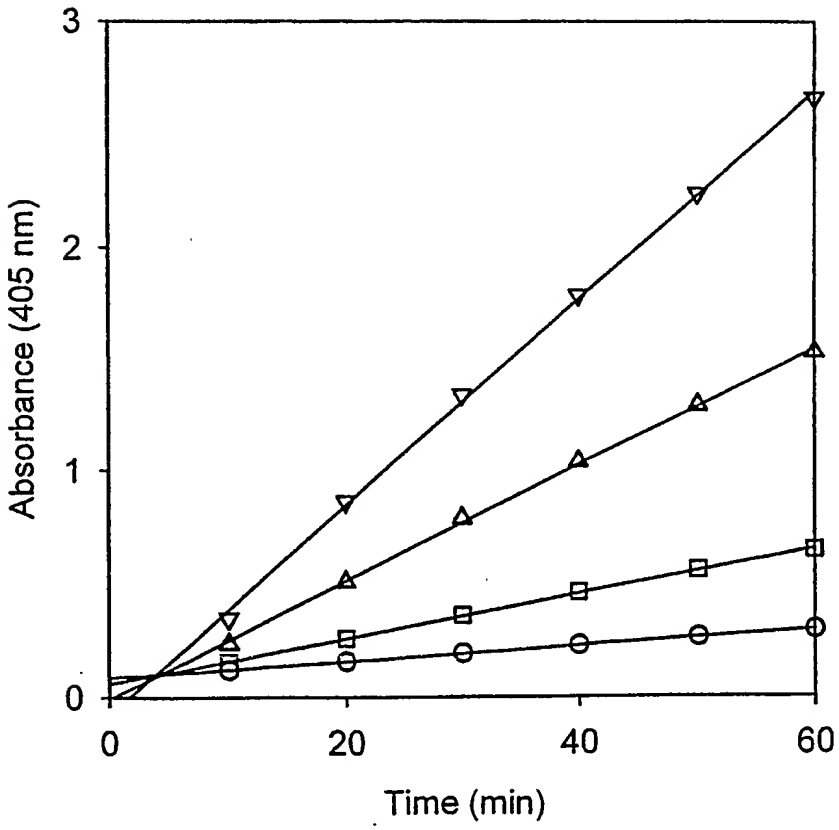


Fig. 1

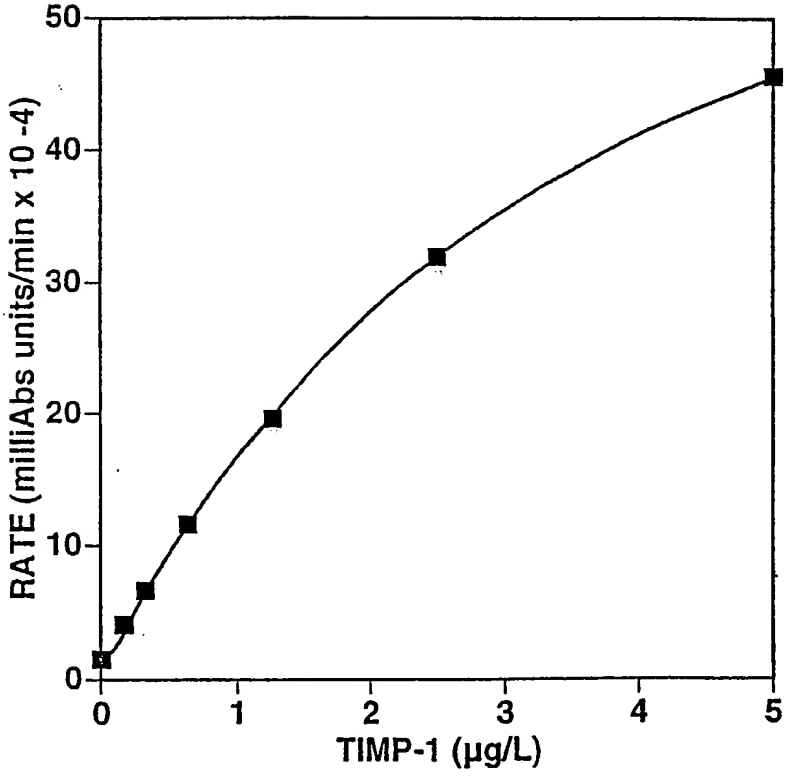


Fig. 2

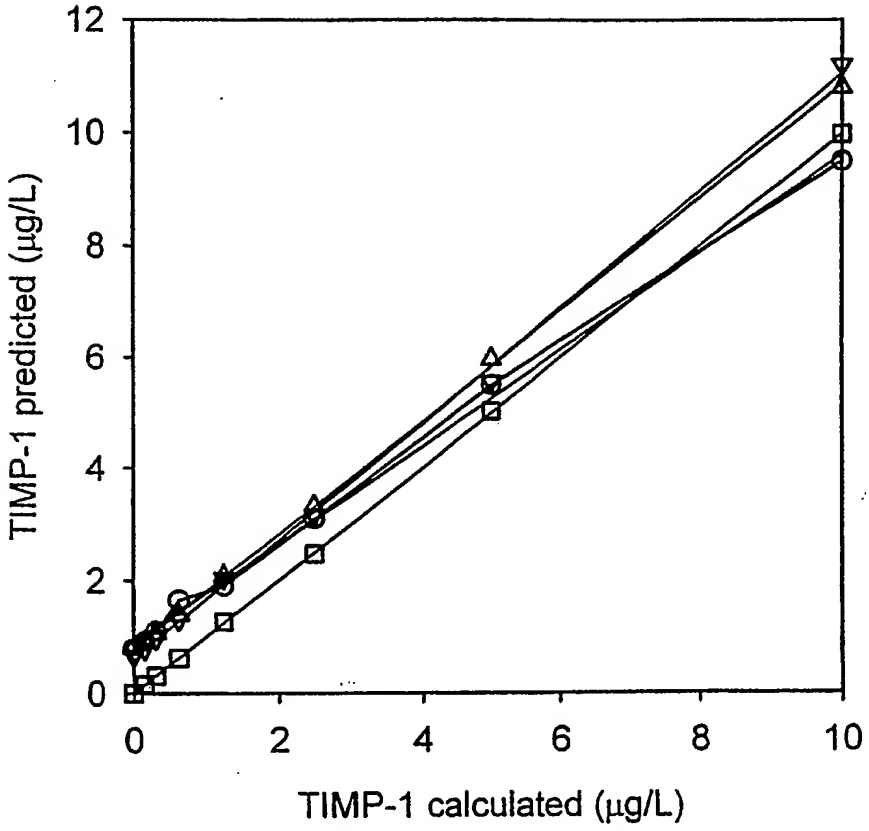

12
10
8
6
4
2
0
0
2
4
6
8
10
TIMP-1 predicted (µg/L)
TIMP-1 calculated (µg/L)

Fig. 3

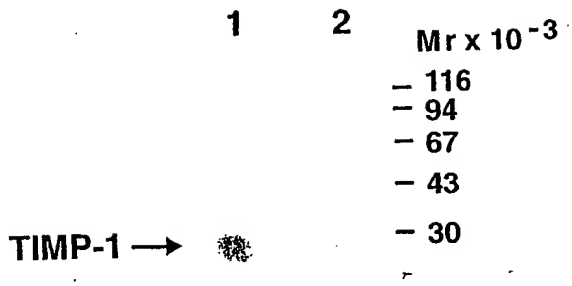


Fig. 4

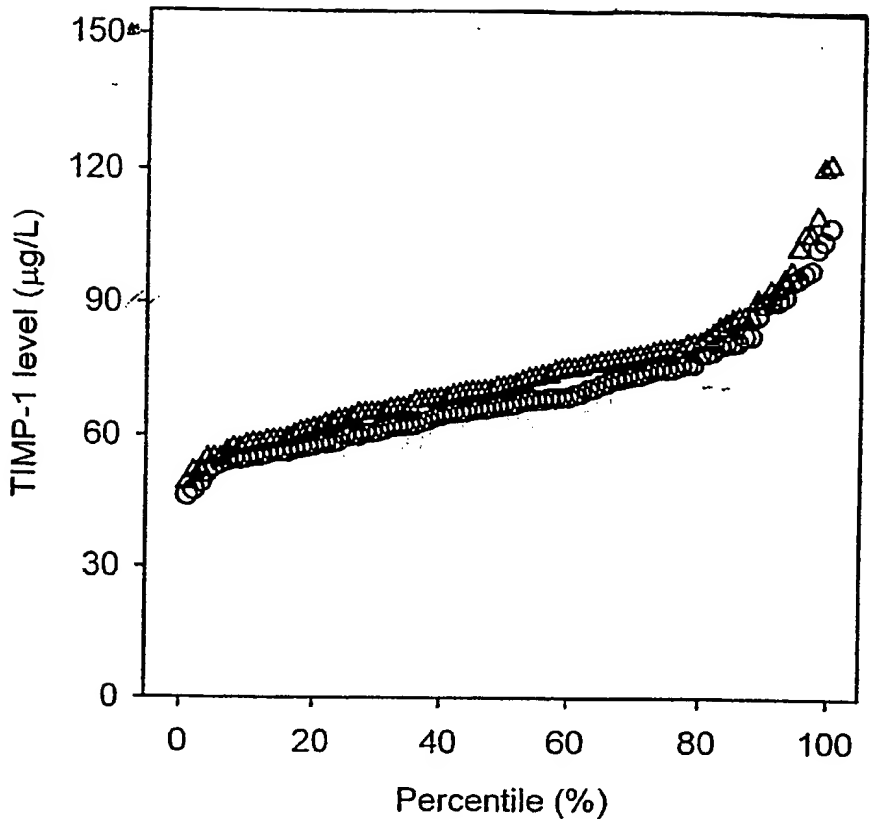

150
120
90
60
30
0
TIMP-1 level (µg/L)
0
20
40
60
80
100
Percentile (%)

Fig. 5a

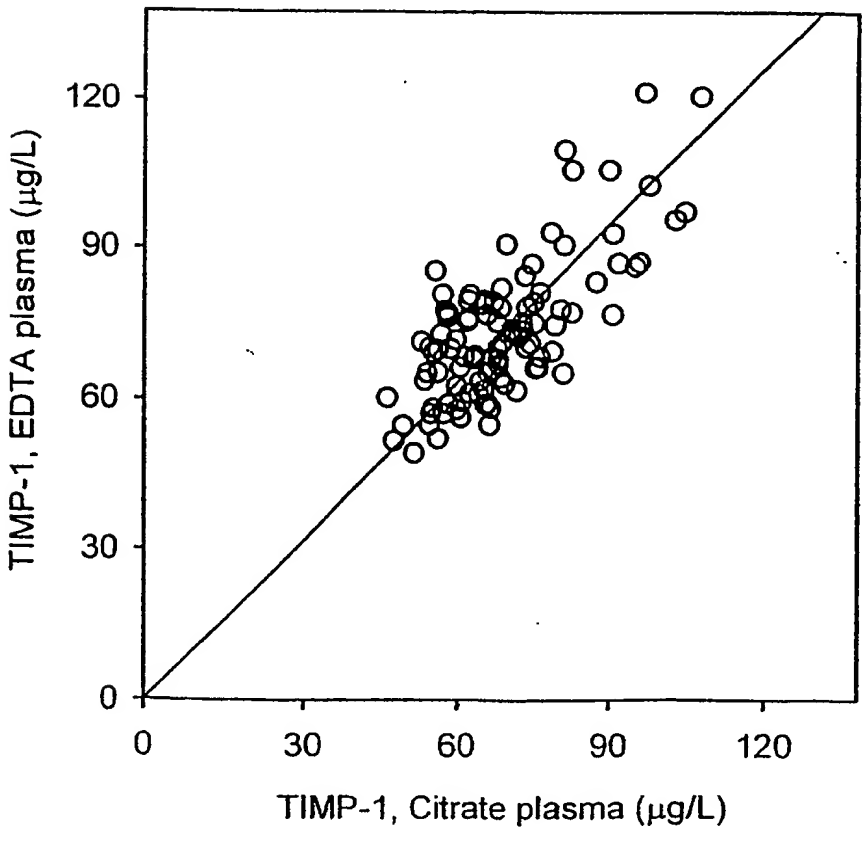

120
90
60
30
0
TIMP-1, EDTA plasma (µg/L)
0
30
60
90
120
TIMP-1, Citrate plasma (µg/L)

Fig. 5b

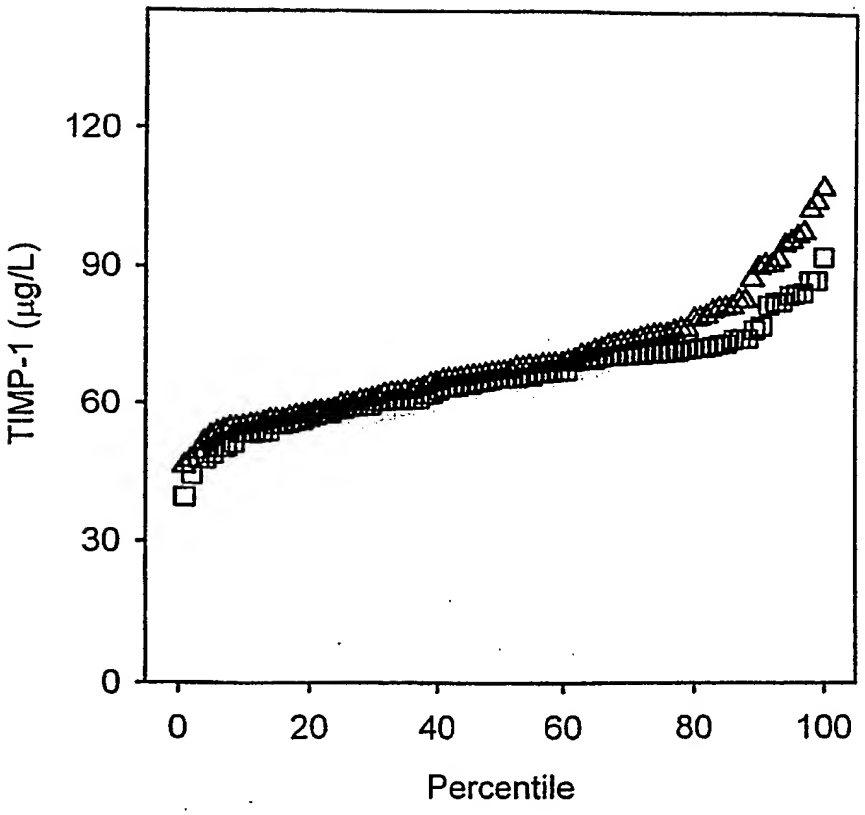


Fig. 6

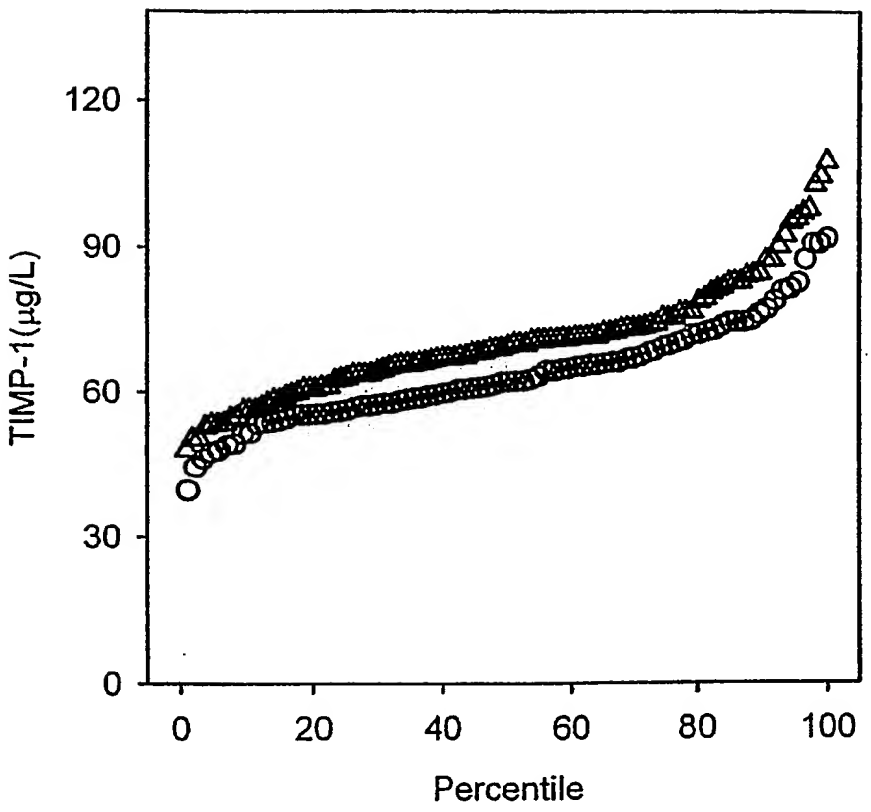


Fig. 7

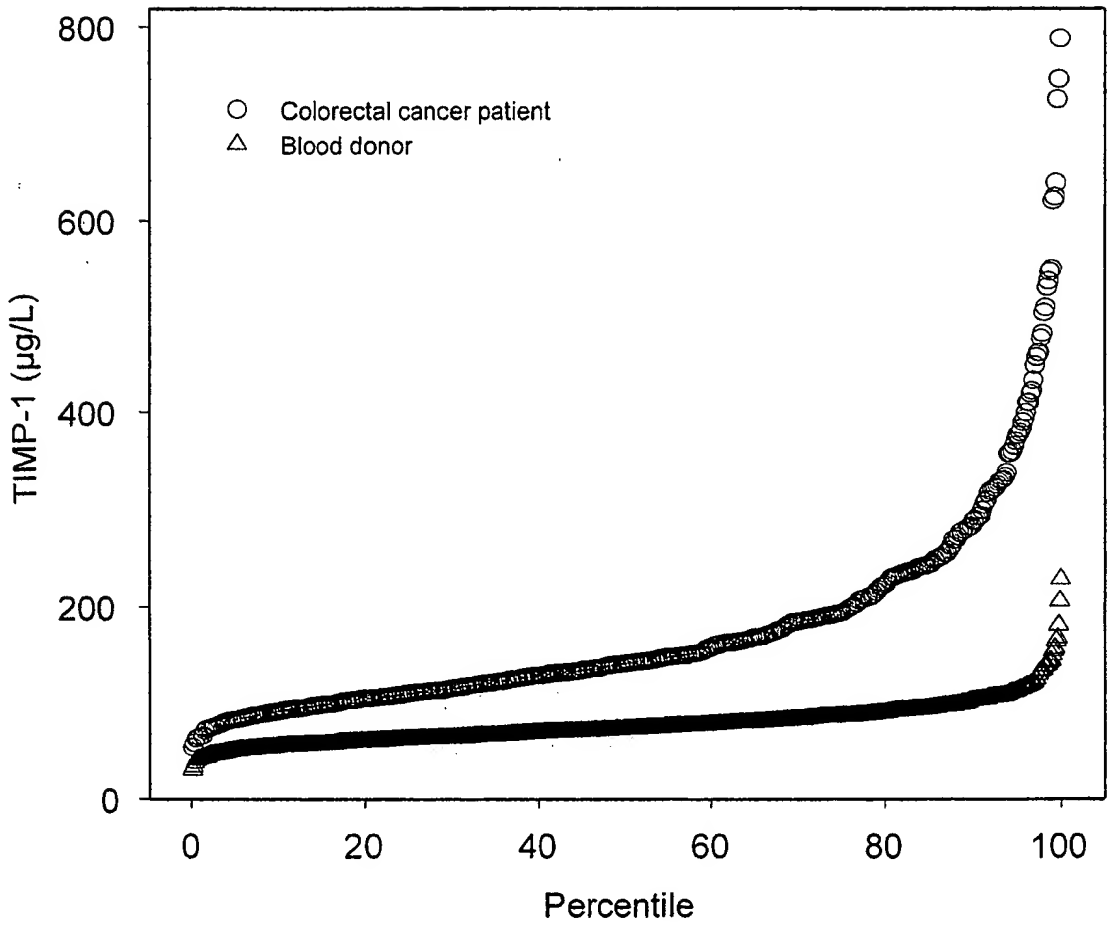

800
600
400
200
0
TIMP-1 (µg/L)
Colorectal cancer patient
Blood donor
0
20
40
60
80
100
Percentile

Fig. 8

ELISA OF HUMAN MMP-9:TIMP-1 IN PLASMA

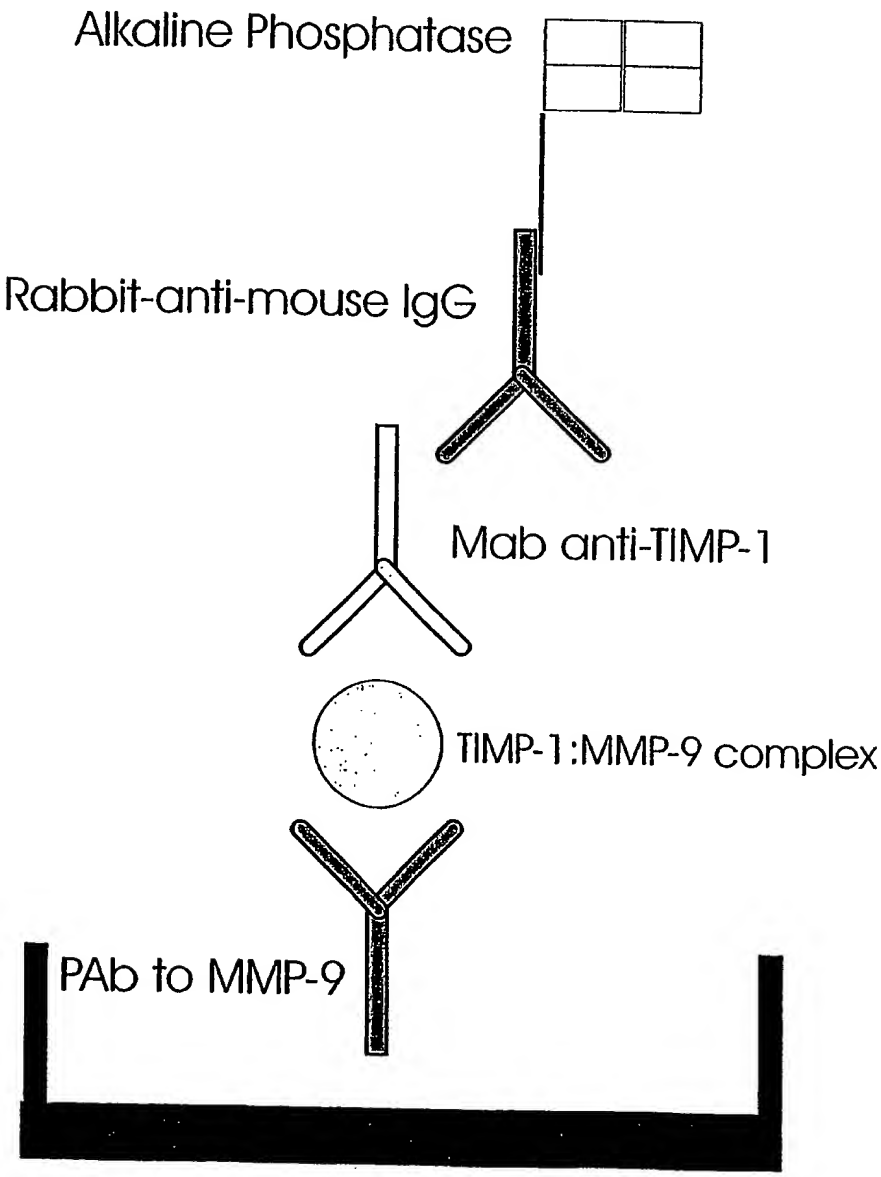


Fig. 9

ELISA OF FREE (NON-COMPLEXED) HUMAN TIMP-1 IN PLASMA

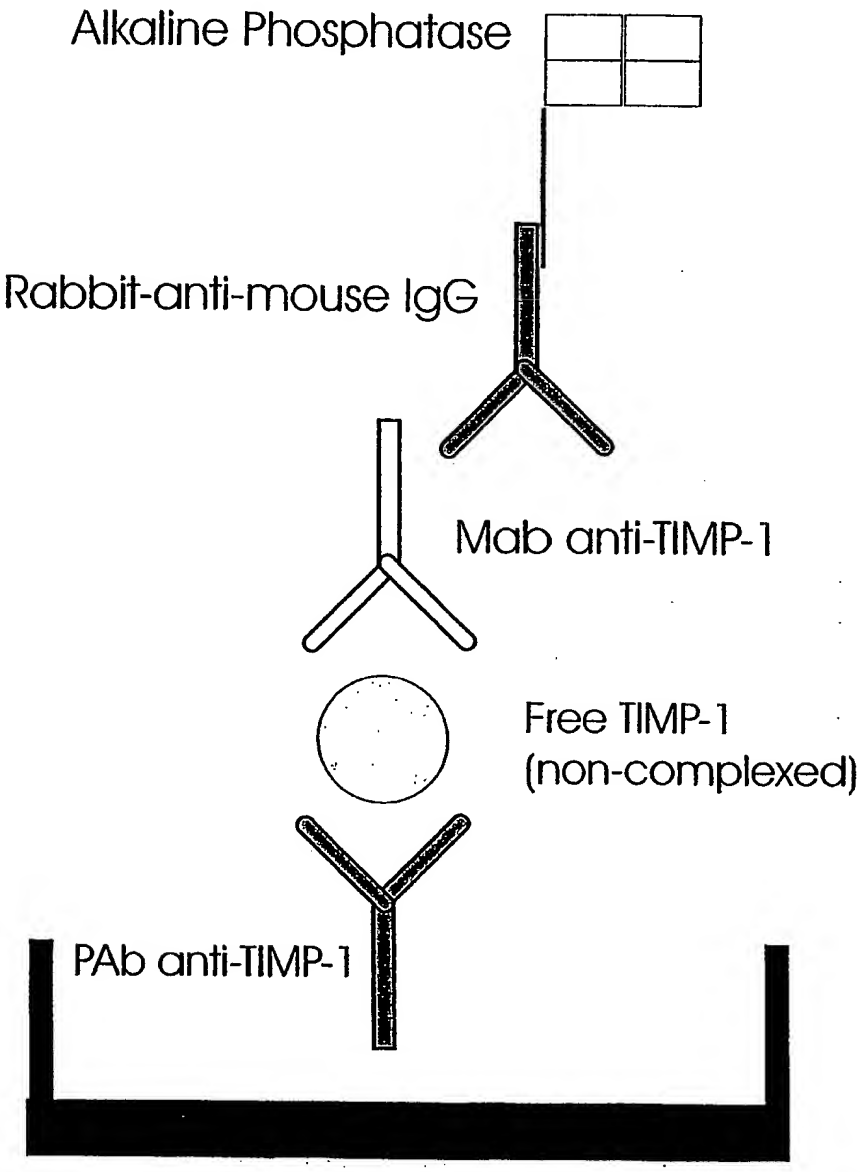


Fig. 10

Antibody tests:
Polyclonal antiMMP9 as catching
MAC19 as detection

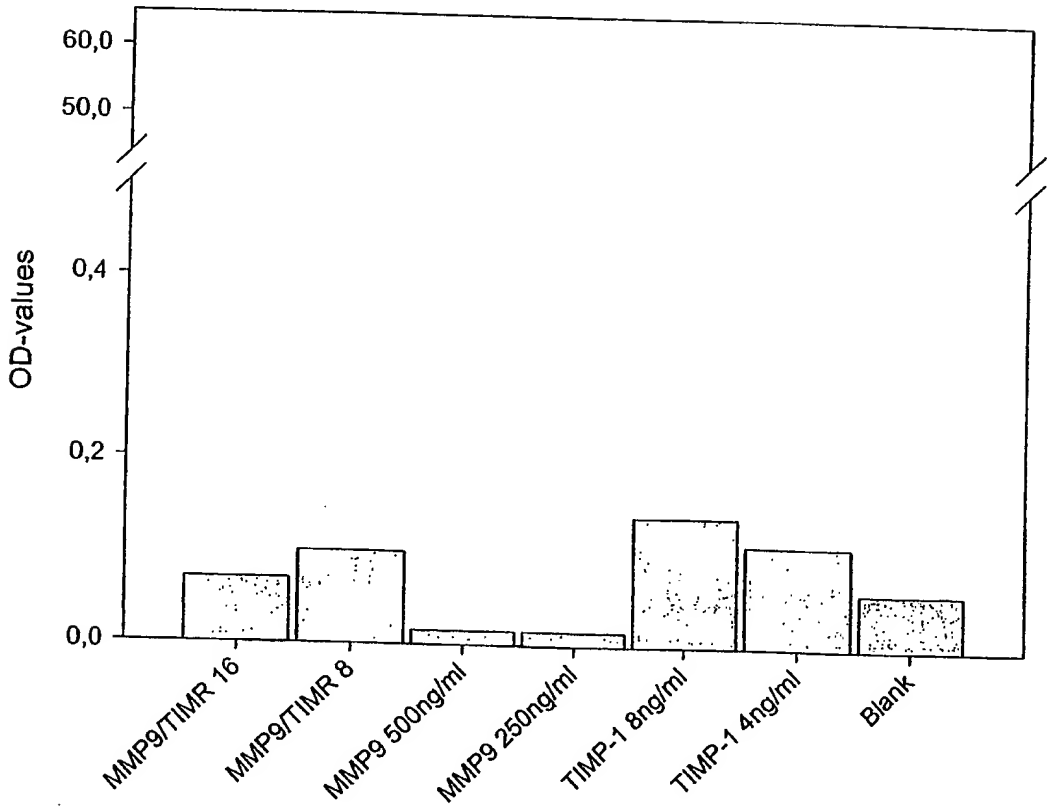


Fig. 11

Antibody tests:
Polyclonal antiMMP9 as catching
MAC15 as detection

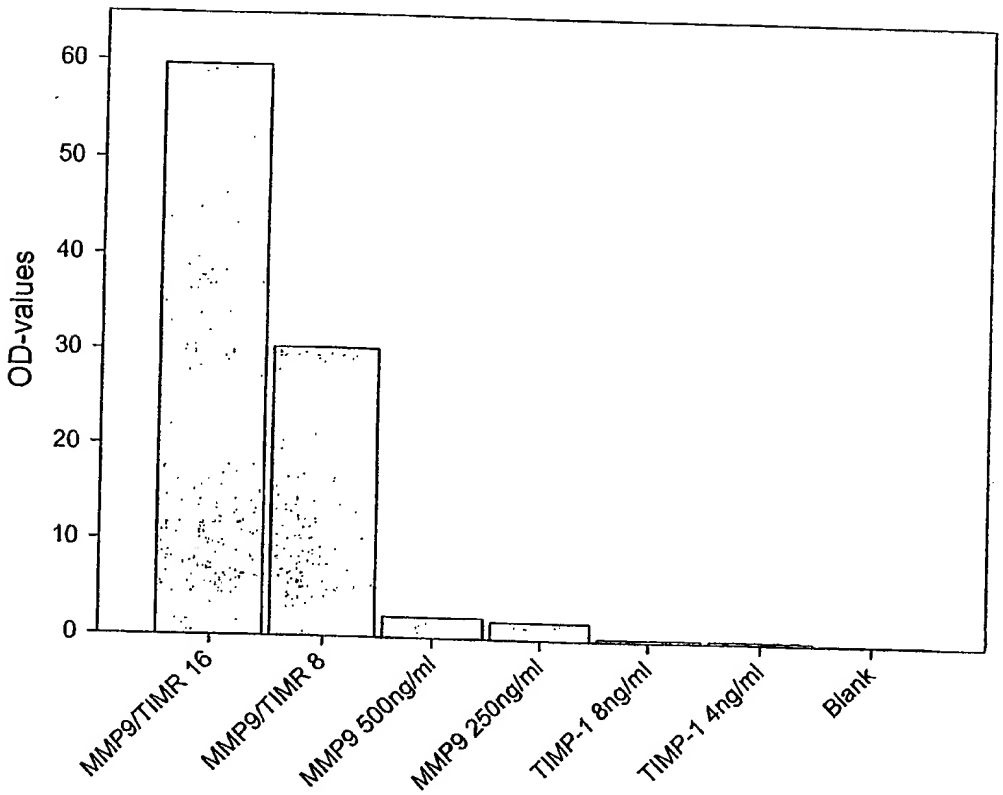
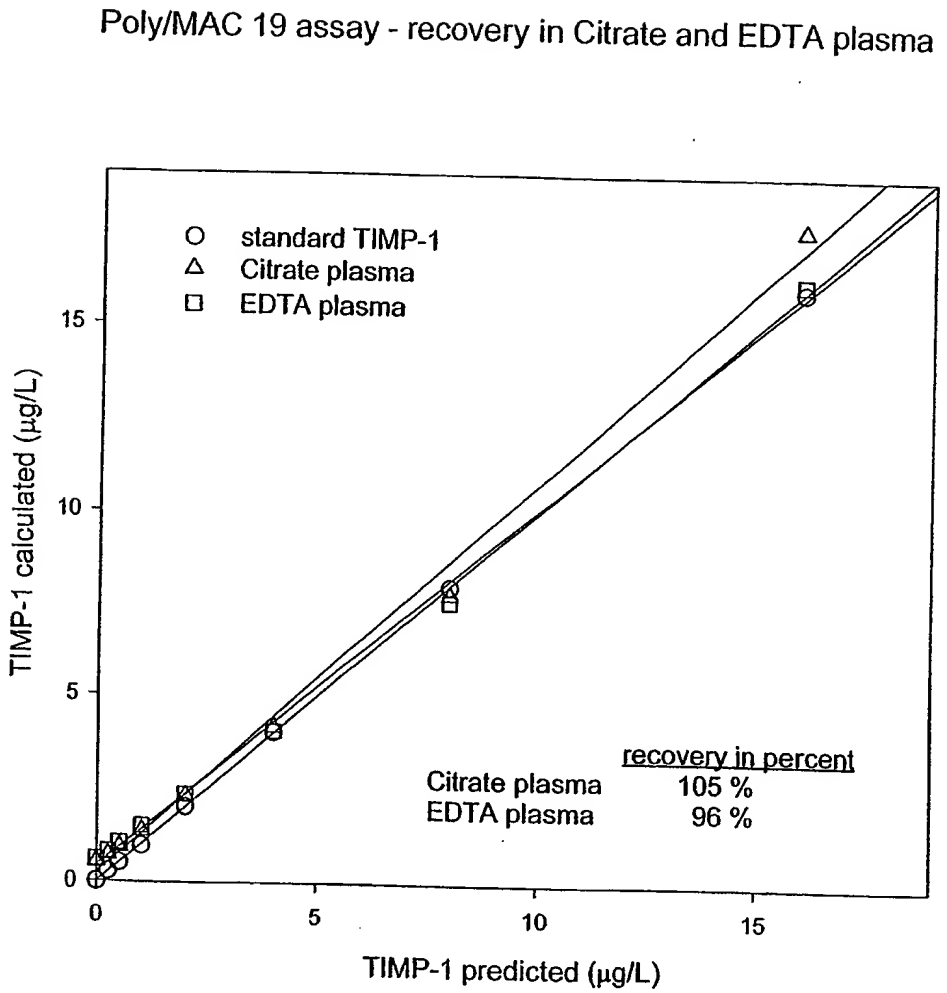


Fig. 12



Intra-assay variation: 9,6% for 24 triplicate measurements of control plasma pool

Fig. 13

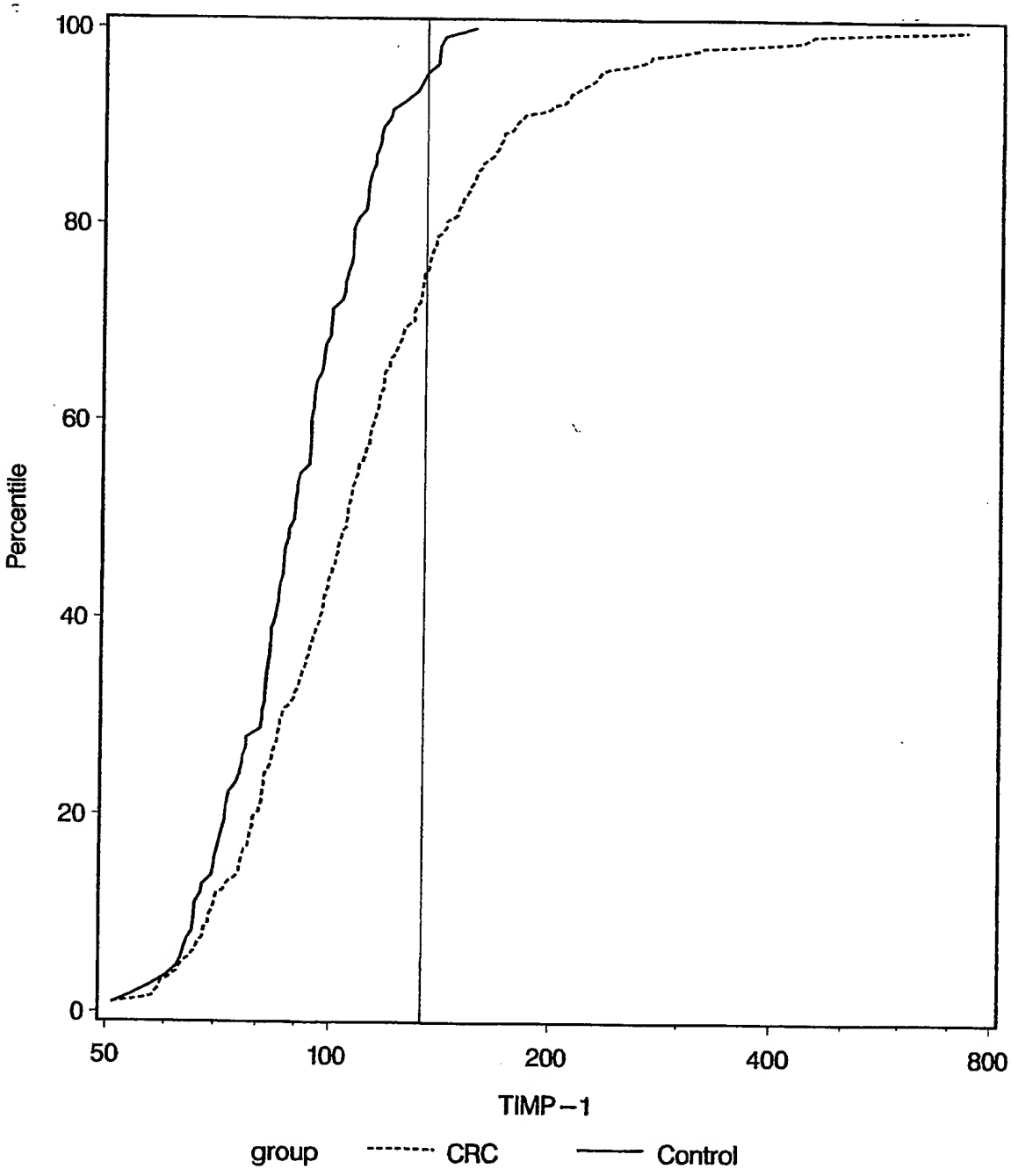
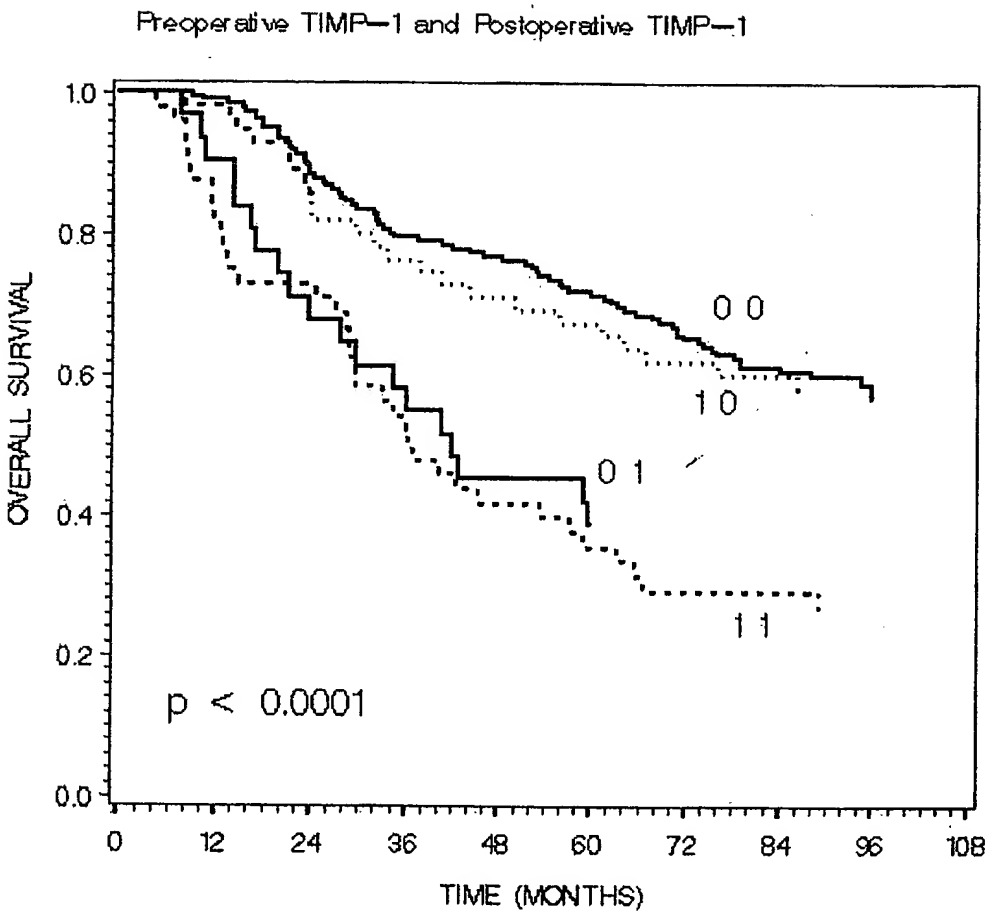
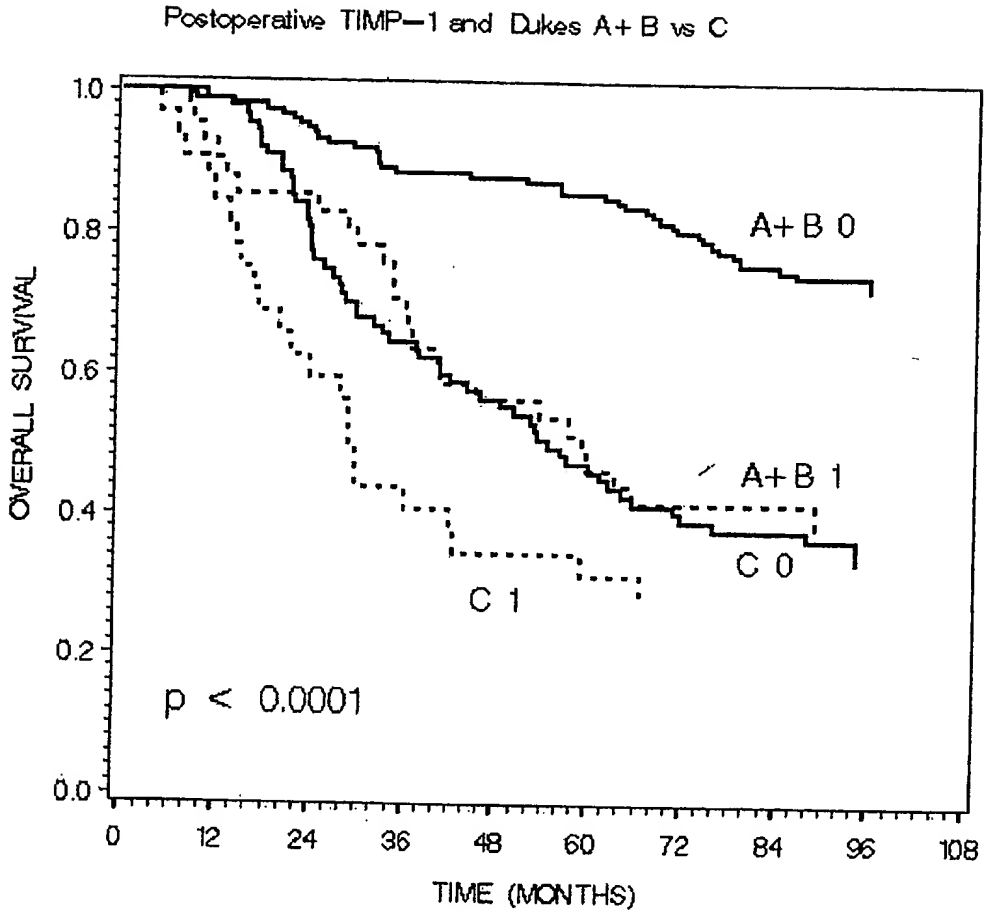

100
80
60
40
20
0
Percentile
50
100
200
400
800
TIMP-1
group
CRC
Control

Fig. 14



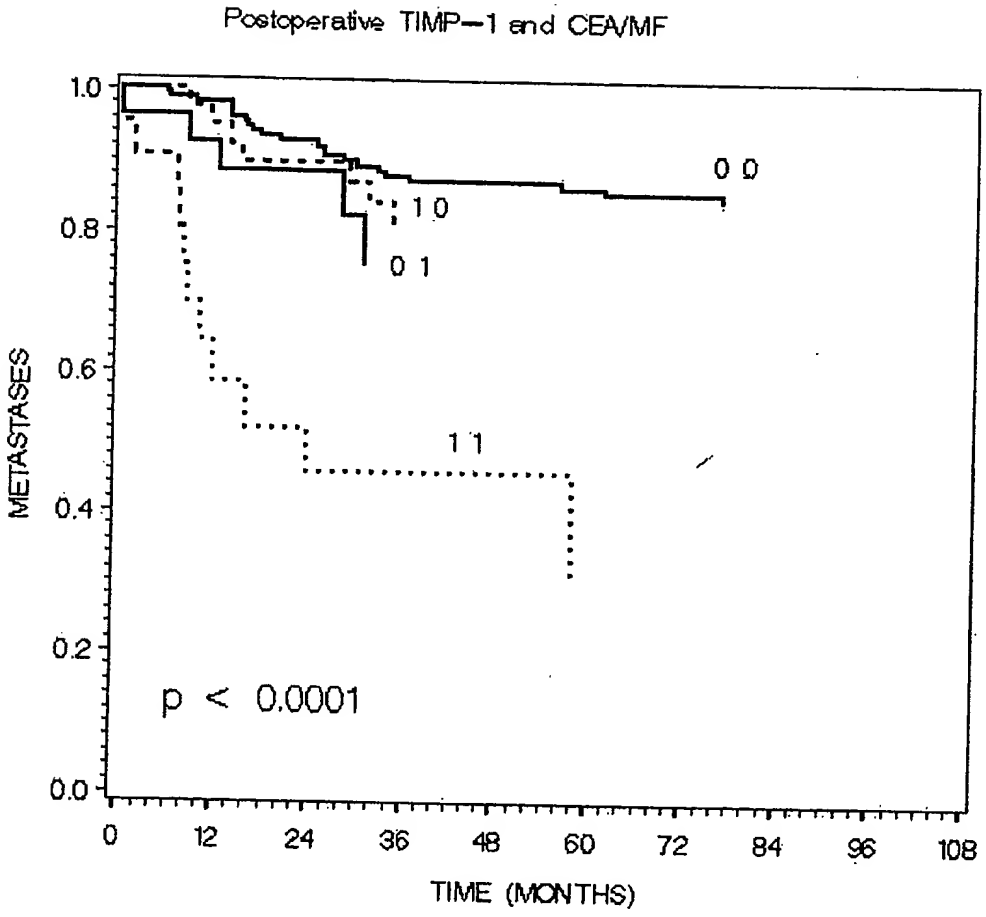
EVENTS	PATIENTS AT RISK				
74	181	160	139	118	0 0
23	55	46	39	34	1 0
19	31	21	14	12	0 1
35	48	35	20	14	1 1

Fig. 15



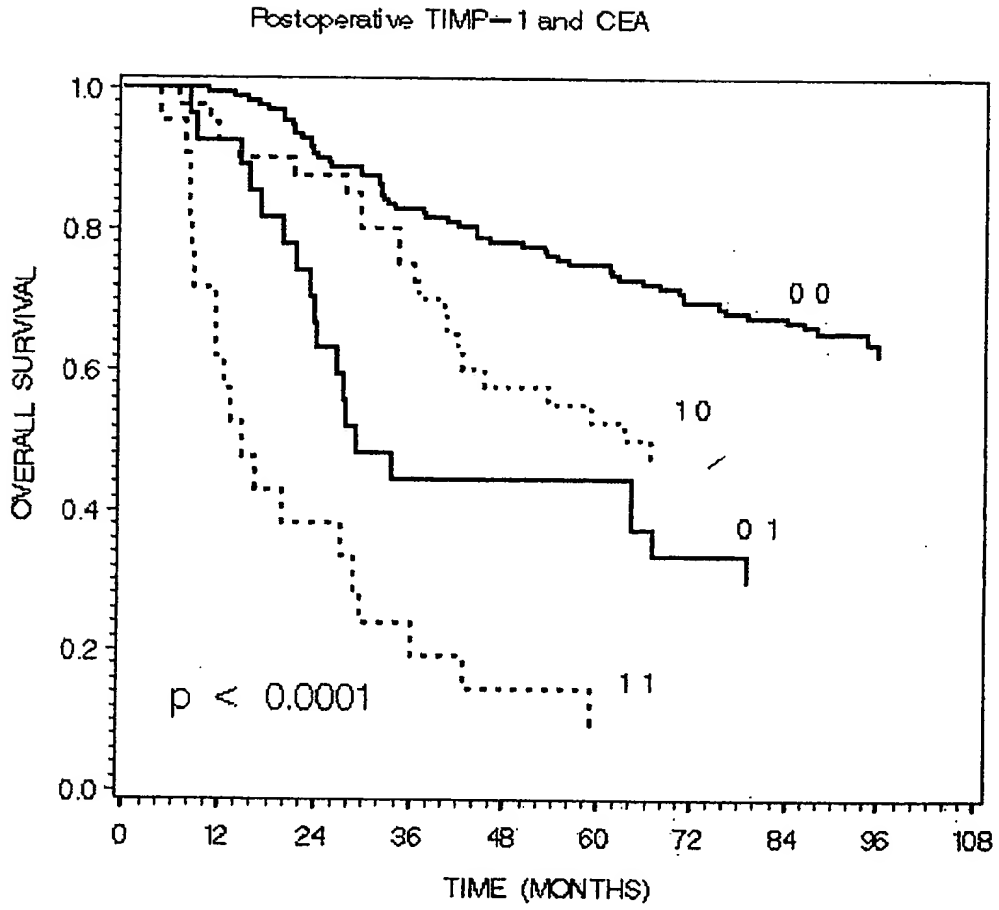
EVENTS	PATIENTS AT RISK				
39	147	138	129	118	A+ B 0
25	41	35	23	17	A+ B 1
56	87	67	49	34	C 0
23	32	19	11	9	C 1

Fig. 16



EVENTS	PATIENTS AT RISK				
22	151	134	116	105	0 0
7	40	34	22	19	1 0
5	27	18	11	8	0 1
11	21	7	3	2	1 1

Fig. 17



EVENTS	PATIENTS AT RISK				
54	151	136	118	105	0 0
21	40	35	23	19	1 0
19	27	18	12	9	0 1
19	21	8	3	2	1 1

Fig. 18

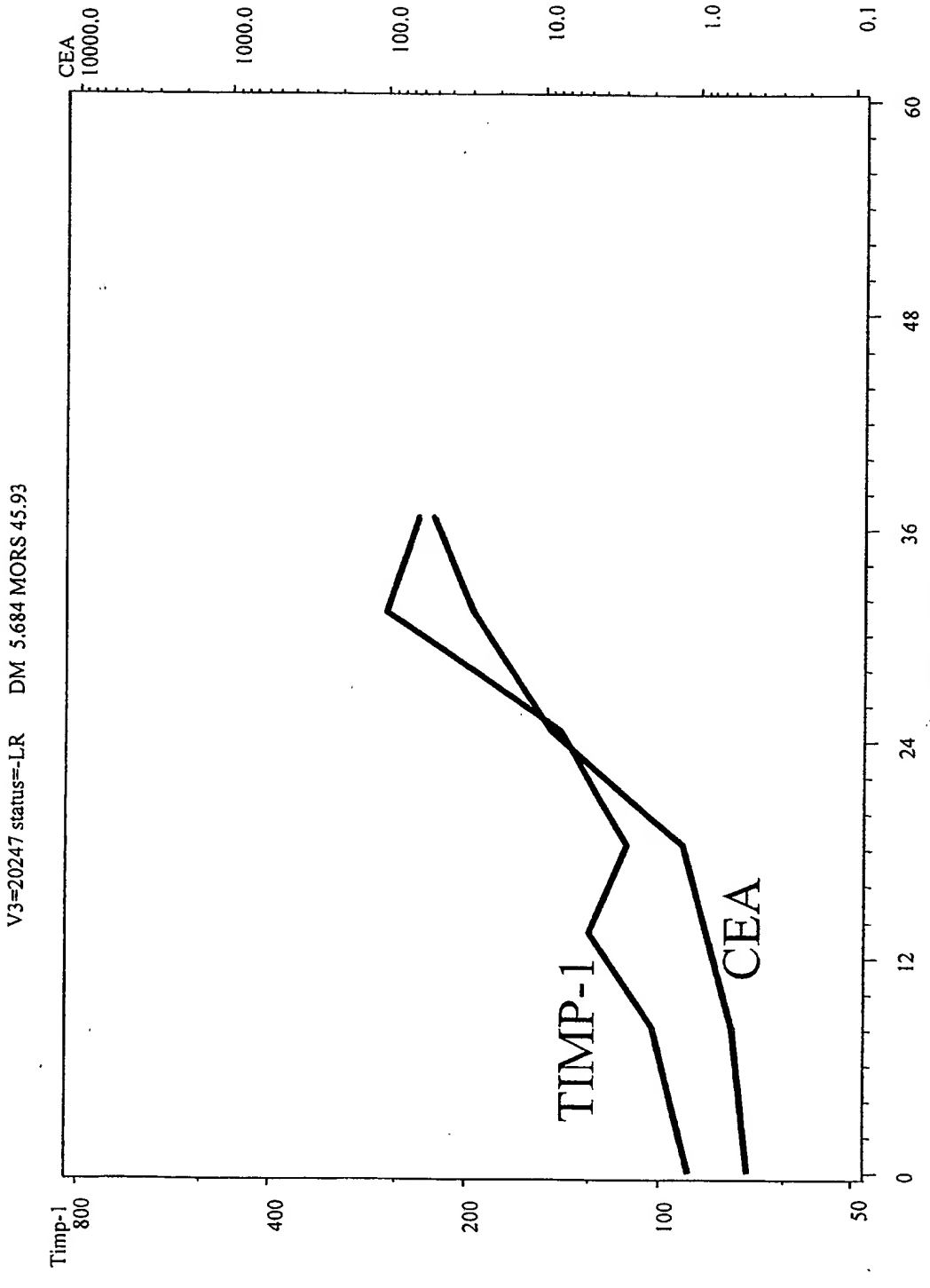

V3=20247 status=-LR DM 5.684 MORS 45.93
Timp-1
800
400
200
100
50
CEA
10000.0
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
Time after operation
TIMP-1
CEA

Fig. 19 A

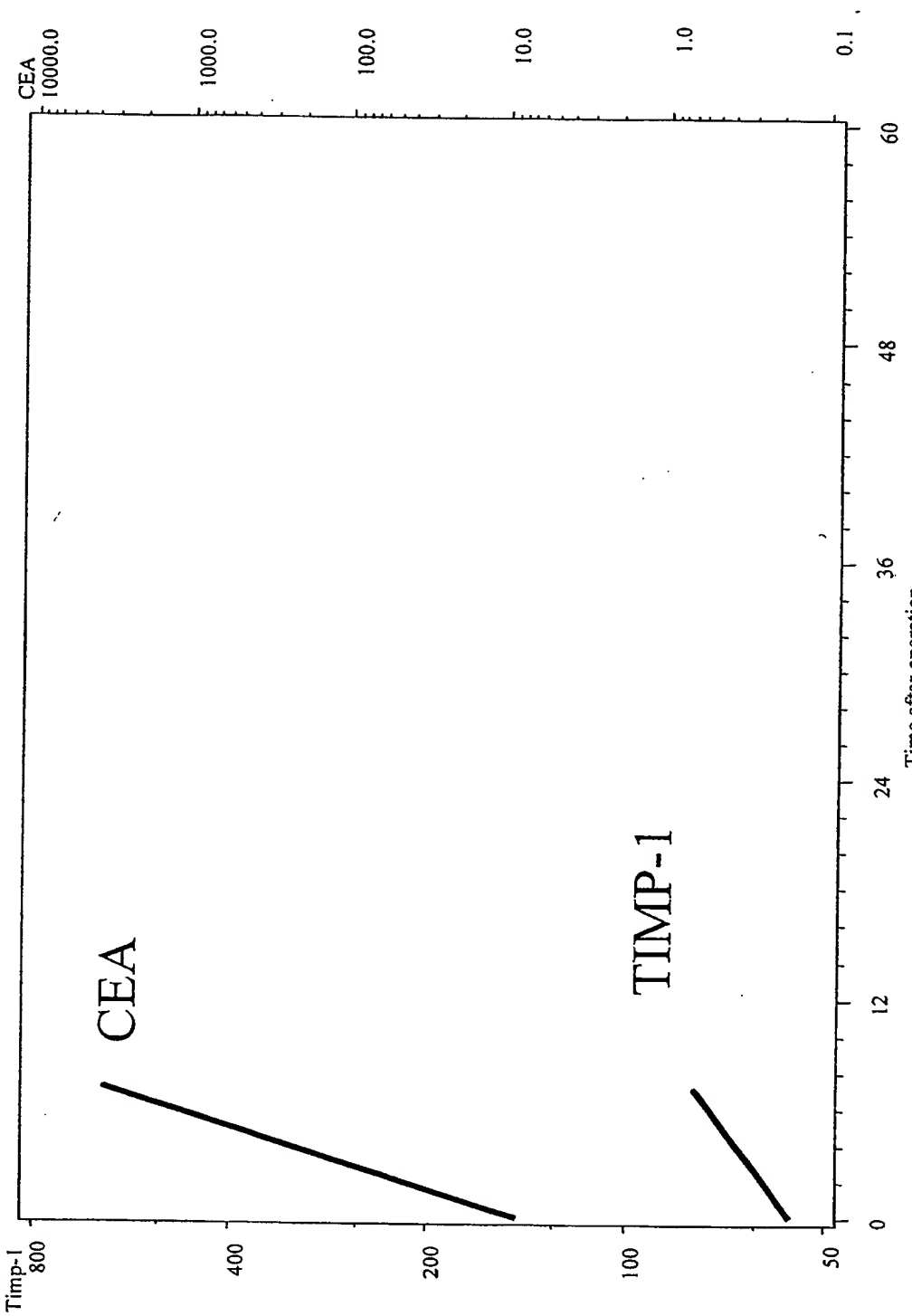

V3=20468 status=LR 16.49 -DM MORS 27.33
CEA
TIMP-1
Timp-1
800
400
200
100
50
CEA
10000.0
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
Time after operation

Fig. 19 B

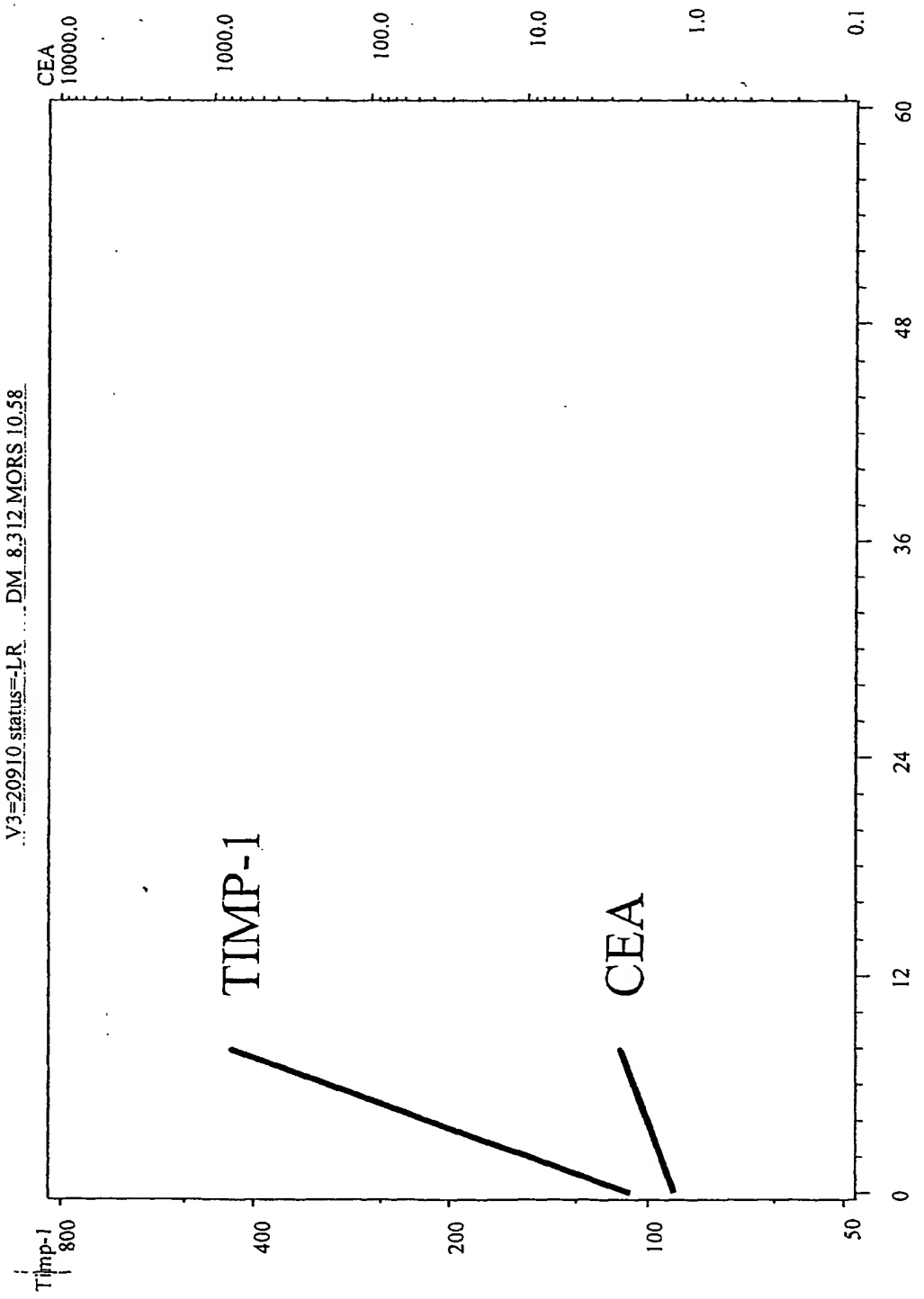

V3=20910 status=-LR DM 8.312 MORS 10.58
CEA
10000.0
1000.0
100.0
10.0
1.0
0.1
Timp-1
800
400
200
100
50
0
12
24
36
48
60
Time after operation
TIMP-1
CEA

Fig. 19 C

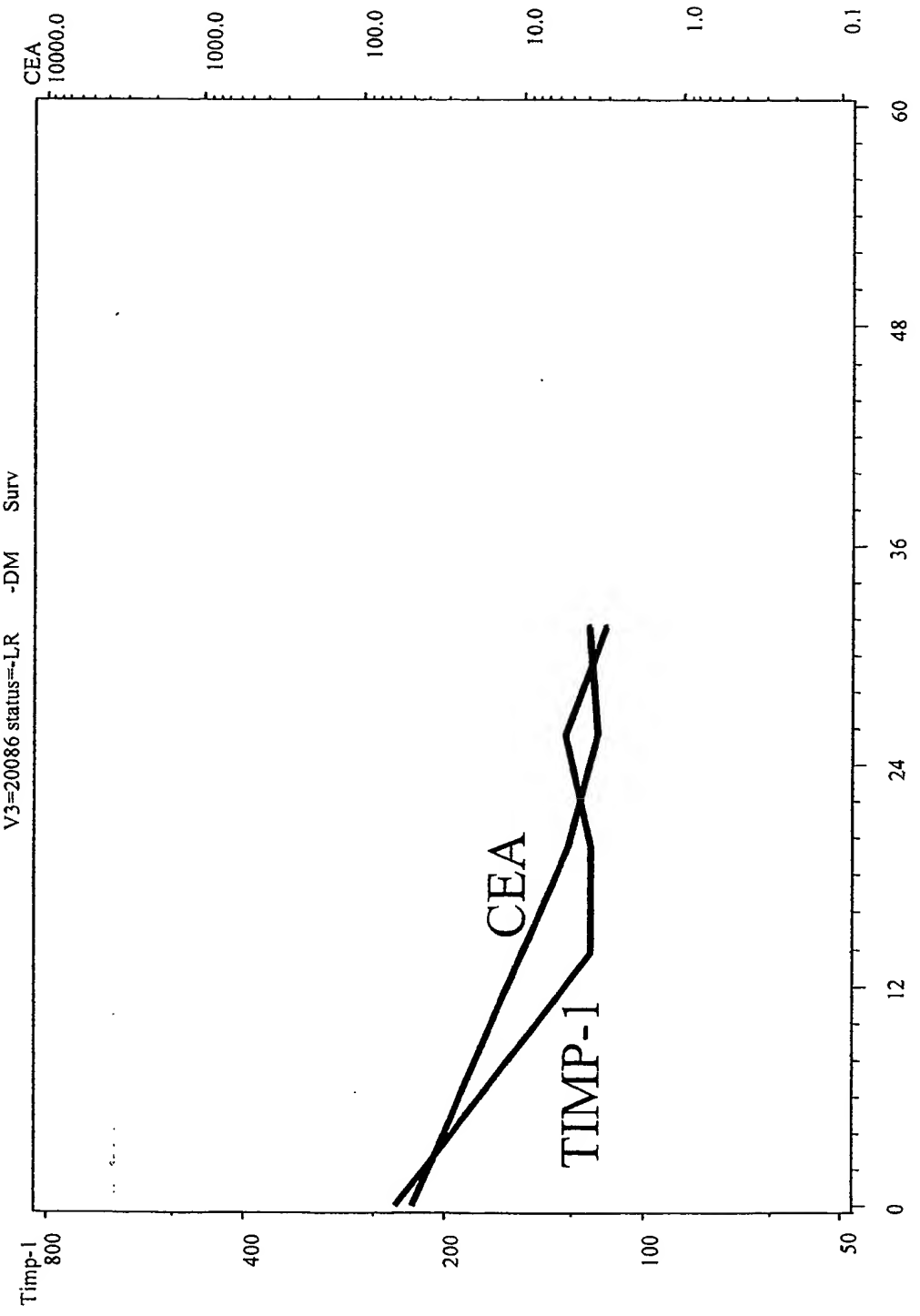

CEA
10000.0
1000.0
100.0
10.0
1.0
0.1
V3=20086 status=-LR -DM Surv
Timp-1
800
400
200
100
50
0
12
24
36
48
60
Time after operation
CEA
TIMP-1

Fig. 19 D